ULTRASTRUCTURAL, PHYSIOLOGICAL, AND CYTOCHEMICAL CHARACTERIZATION OF CORE-LIKE STRUCTURES IN GROUP D STREPTOCOCCI

By

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TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	ii
LIST OF TABLES	v
LIST OF FIGURES	vi
ABSTRACT	xvi
INTRODUCTION	1
LITERATURE REVIEW	5
MATERIALS AND METHODS	28
RESULTS	42
DISCUSSION	102
LITERATURE CITED	114
BIOGRAPHICAL SKETCH	127

LIST OF TABLES

Table		Page
1.	Conditions for the Enzymic Digestion of Cores from Thin Sections of *Streptococcus faecalis* Strain XL	33
2.	Comparison of the Effects of Chemical Fixation on the Morphology of Cores in Group D *Streptococci*	43
3.	Diameters of Cores from *Streptococcus faecalis* ATCC 8043 and *Streptococcus faecalis* Strain XL	55
4.	Observation of the Formation of Mesosomes and Cores in Cells of *Streptococcus faecalis* Strain XL During Growth in Todd-Hewitt Broth at 37 C	58
5.	Determination of Colony-Forming-Units (CFU) in Relation to the Formation of Cores in *Streptococcus faecalis* Strain XL Growing in Todd-Hewitt Broth at 37 C	62
6.	Changes in Cells of *Streptococcus faecalis* Strain XL With and Without Cores After Inoculation Into Fresh and Spent Todd-Hewitt Broth at 37 C	72
7.	Composition of Cell Walls of *Streptococcus faecalis* Strain XL	98

LIST OF FIGURES

Figure		Page
1.	Longitudinal section of a core in *Streptococcus faecalis* strain XL fixed for 30 min in 2% glutaraldehyde in the cold and for 3 hr in 1% osmium tetroxide at room temperature	46
2.	Freeze-etched, unfixed cell of *S. faecalis* Strain XL after growth for 12 hr in Todd-Hewitt broth at 37 C	46
3.	Freeze-etched, unfixed cell of *S. faecalis* strain XL after growth for 12 hr in Todd-Hewitt broth at 37 C	46
4.	Freeze-etched cell of *S. faecalis* strain XL fixed for 2 hr in 2% glutaraldehyde in the cold	46
5.	Thin section of *S. faecalis* ATCC 8043 fixed for 30 min with 2% glutaraldehyde in the cold and for 3 hr in 1% OsO_4 at room temperature	48
6.	Freeze-etched, unfixed cell of *S. faecalis* ATCC 8043 showing a longitudinal fracture through a core	48
7.	Cross section of a core in a thin section of *S. faecalis* ATCC 8043 fixed for 30 min with 2% glutaraldehyde in the cold and for 3 hr in 1% OsO_4 at room temperature	48
8.	Freeze-etched, unfixed cell of *S. faecalis* ATCC 8043 showing a cross section of a core	48

LIST OF FIGURES—*Continued*

Figure		Page
9.	Freeze-etched, unfixed cell of *S. faecalis* strain XL showing the surface of a core	51
10.	Fracture through the membrane of an unfixed, freeze-etched cell of *S. faecalis* strain XL showing a round configuration (arrow) thought to represent the end of a core	51
11.	Thin section of a cell of *S. faecalis* strain XL fixed by the Ryter-Kellenberger technique with 1% OsO_4 for 16 hr at room temperature	51
12.	Thin section of a cell of *S. faecalis* strain XL cultured for 24 hr at 26 C and fixed for 30 min with 2% glutaraldehyde in the cold	51
13.	Isolated core-like structures obtained from the disintegration of cells of *S. faecalis* strain XL in the Braun homogenizer after fixation for 30 min with 4% glutaraldehyde in the cold	54
14.	Isolated core-like structures obtained from the disintegration of cells of *S. faecalis* strain XL in the Braun homogenizer after fixation for 30 min with 4% glutaraldehyde in the cold	54
15.	Isolated core-like structures obtained from the disintegration of cells of *S. faecalis* strain XL in the Braun homogenizer while suspended in 0.1 M HCl-KCl buffer, pH 2.0	54
16.	Growth of *Streptococcus faecalis* strain XL in Todd-Hewitt broth at 37 C, showing changes in (1) dry weight and (ii) optical density	57

Figure | Page

17. Thin section of cells of *S. faecalis* XL after growth for 1 hr in Todd-Hewitt medium at 37 C 60

18. Thin section of cells of *S. faecalis* strain XL after growth for 2½ hr in Todd-Hewitt broth at 37 C 60

19. Thin section of a cell of *S. faecalis* strain XL after growth for 3 hr in Todd-Hewitt broth at 37 C 60

20. Thin section of a cell of *S. faecalis* strain XL after growth for 4 hr in Todd-Hewitt broth at 37 C 60

21. Thin section of cells of *S. faecalis* strain XL after growth for 13½ hr in Todd-Hewitt broth at 37 C 60

22. Thin section of *S. faecalis* strain XL after growth for 20 hr in Todd-Hewitt broth at 37 C 60

23. Growth of a culture of *Streptococcus faecalis* strain XL in Todd-Hewitt broth at 37 C for 7 days (168 hr) for the observation of the persistence of cores and mesosomes in cells in old cultures 61

24. Growth of cultures of *Streptococcus faecalis* strain XL in Todd-Hewitt broth at 37 C after addition of 50 μg of chloramphenicol: (A) 1½ hr before the normal time for formation of cores, and (B) 30 min before this time 64

25. Thin section of control cells of *S. faecalis* strain XL grown to mid-log phase in Todd-Hewitt broth at 37 C and fixed for 30 min with 2% glutaraldehyde in the cold 66

LIST OF FIGURES—*Continued*

Figure		Page
26.	Thin section of cells of *S. faecalis* strain XL from a culture to which 50 μg of chloramphenical (CAP) per ml was added for 1½ hr	66
27.	Thin section of a cell of *S. faecalis* strain XL from a culture to which 50 μg of CAP per ml was added for 1½ hr after growth for 2½ hr	66
28.	Thin section of a control cell of *S. faecalis* strain XL from a culture in Todd-Hewitt broth incubated for 4 hr at 37 C and fixed for 30 min with 2% glutaraldehyde in the cold	68
29.	Thin section of a cell of *S. faecalis* strain XL exposed to 50 μg of CAP per ml for 1½ hr after growth for 2½ hr in Todd-Hewitt broth at 37 C and subsequent culturing for 24 hr at 37 C in fresh Todd-Hewitt broth	68
30.	Same as Fig. 29 except that the cells were cultured subsequently for 24 hr in spent Todd-Hewitt broth, pH 5.9, which was filtered from a stationary culture	68
31.	Thin section of cells of *S. faecalis* strain XL from a culture growing in Todd-Hewitt broth to which 50 μg of CAP per ml was added 30 min prior to the time of normal formation of cores	68
32.	Thin section of control cells of *S. faecalis* strain XL cultured for 5 hr in Todd-Hewitt broth at 37 C	71
33.	Thin section of a cell of *S. faecalis* strain XL before introduction into fresh Todd-Hewitt broth	71

Figure Page

34. Thin section of cells of *S. faecalis* strain XL 5 min after introduction into fresh Todd-Hewitt broth, pH 7.2 at 37 C 71

35. Thin section of a cell of *S. faecalis* strain XL cultured for 16 hr in Todd-Hewitt broth + 1% glucose at 37 C and then incubated for 60 min at 2 C 71

36. Growth of cultures of *Streptococcus faecalis* strain XL in (A) Todd-Hewitt broth, unbuffered; (B) Todd-Hewitt broth buffered with 0.01 M citrate buffer, pH 4.5; and (C) Todd-Hewitt broth buffered with 0.01 M Tris buffer, pH 7.9 74

37. Thin section of control cells of *S. faecalis* strain XL cultured for 14 hr in unbuffered Todd-Hewitt broth at 37 C to a final pH of 5.6 76

38. Thin section of cells of *S. faecalis* ATCC 8043 cultured for 16 hr at 37 C in Todd-Hewitt broth buffered with 0.02 M potassium phosphate buffer, pH 7.4 76

39. Thin section of cells of *S. faecalis* ATCC 8043 which were cultured for 16 hr at 37 C in Todd-Hewitt broth buffered with 0.02 M potassium phosphate buffer, pH 7.4 and then incubated for an additional 1½ hr at 37 C after the pH of the medium was lowered to 4.4 76

40. Thin section of cells of *S. faecalis* strain XL cultured for 4½ hr at 37 C in Todd-Hewitt broth buffered with 0.01 M Tris buffer, pH 7.9 76

Figure Page

41. Thin section of a cell of *S. faecalis* strain XL cultured for 4½ hr at 37 C in Todd-Hewitt broth buffered with 0.01 M citrate buffer, pH 4.5 for a final pH of 4.73 79

42. Thin section of cells of *S. faecalis* ATCC 8043 incubated for 60 min in 0.1 M HCl-KCl buffer, pH 2.0 79

43. Thin section of a cell of *S. faecalis* ATCC 8043 incubated for 60 min in 0.1 M citrate buffer, pH 4.0 79

44. Thin section of cells of *S. faecalis* ATCC 8043 incubated for 30 min in distilled water at pH 6.0 79

45. Thin section of cells of *S. faecalis* ATCC 8043 in Todd-Hewitt broth after the pH was raised from 6.1 to 7.2, following the induction of cores, and the culture incubated for an additional 30 min at 37 C 81

46. Thin section of a cell of *S. faecalis* ATCC 8043 taken from a culture in stationary growth after the formation of cores and incubated for 30 min at 37 C in 0.1 M Tris buffer, pH 7.2 81

47. Thin section of a cell of *S. faecalis* ATCC 8043 taken from a culture in stationary growth phase after the formation of cores and incubated for 30 min at 37 C in 0.1 M Tris buffer, pH 8.0 81

Figure Page

48. Thin section of cells of *S. faecalis* ATCC 8043 cultured to the stationary growth phase after which the pH of the medium was raised to 8.0, and the culture incubated for an additional 30 min at 37 C 81

49. Thin section of a cell of *S. faecalis* ATCC 8043 cultured to the stationary phase at pH 5.9 after which the pH of the medium was raised to 8.0 and the culture incubated for an additional 30 min at 2 C 84

50. Thin section of core-containing cell of *S. faecalis* ATCC 8043 washed for 5 min with 0.1 Tris buffer, pH 8.2 in 0.5 M sucrose 84

51. Thin section of a young cell of *S. faecalis* ATCC 8043 taken from a logarithmically growing culture and permitted to autolyze for 30 min in 0.01 M potassium phosphate buffer, pH 6.5, at 37 C 84

52. Thin section of a young cell of *S. faecalis* ATCC 8043 taken from a logarithmically growing culture and permitted to autolyze for 30 min in 0.01 M potassium phosphate buffer, pH 6.5, at 37 C 84

53. Thin section of cells of *S. faecalis* strain XL from a logarithmically growing culture after autolysis for 30 min at 37 C in 0.01 M phosphate buffer, pH 6.5 86

54. Thin section of a cell of *S. faecalis* strain XL from a logarithmically growing culture after autolysis for 30 min at 37 C in 0.01 M phosphate buffer, pH 6.5 86

Figure Page

55. Thin section of a cell of *S. faecalis* strain XL from a logarithmically growing culture after autolysis for 30 min at 37 C in 0.01 M phosphate buffer, pH 6.5 86

56. Thin section of cells of *S. faecalis* strain XL from a logarithmically growing culture 30 min after the addition of 1000 units of penicillin G per ml 86

57. Thin section of a spheroplast of *S. faecalis* strain XL formed by exposure to 250 μg of lysozyme per ml in distilled water at pH 6.0 for 30 min at 37 C 89

58. Thin section of a spheroplast of *S. faecalis* strain XL formed by exposure at 250 μg of lysozyme per ml in distilled water for 30 min at 37 C 89

59. Typical morphology of a mesosome in a thin section of a spheroplast of *S. faecalis* strain XL formed by exposure to 250 μg of lysozyme per ml for 30 min at 37 C in distilled water, pH 6.0 89

60. Longitudinal section of an apparently disintegrating core in a spheroplast of *S. faecalis* strain XL formed by exposure to 250 μg of lysozyme per ml for 30 min at 37 C in distilled water, pH 6.0 89

61. Freeze-etched, unfixed spheroplast of *S. faecalis* strain XL showing the structure of the core 91

62. Freeze-etched, unfixed spheroplast of *S. faecalis* strain XL showing the structure of the core 91

Figure Page

63. Freeze-etched, unfixed spheroplast of *S. faecalis* strain XL showing the structure of the core 91

64. Freeze-etched, unfixed, young cell of *S. faecalis* strain XL showing the typical morphology of a mesosome in contrast to the cross section of a core 91

65. Freeze-etched, unfixed cell of *S. faecalis* strain XL showing a formation that may represent an intermediate form in the formation of cores 94

66. Thin section of cells of *S. faecalis* strain XL after treatment for 30 min with 250 μg of lysozyme per ml at 37 C in distilled water at pH 6.0 followed by lysis in the mixture Dirksen used for stabilizing microtubules: 0.1 M EDTA, 0.5 M sucrose, and 0.15 M dithiodipropanol 94

67. Thin section of spheroplasts of *S. faecalis* strain XL after treatment with 250 μg of lysozyme per ml for 30 min at 37 C in 0.01 M Tris buffer, pH 8.4 94

68. Thin section of cells of *S. faecalis* ATCC 8043 in which unusual crystalline-like inclusions can be seen 94

69. Thin section of *S. faecalis* strain XL stained for 20 min by the silver methenamine staining procedure 97

70. Longitudinal section of *S. faecalis* strain XL stained for 20 min by the silver methenamine staining procedure 97

Figure Page

71. Cross section of a core in a thin section of *S. faecalis* strain XL stained for 20 min by the silver methenamine staining procedure 97

72. Thin section of cells of *S. faecalis* strain XL stained for 20 min by the silver methenamine staining procedure 97

73. Thin section of a control cell of *S. faecalis* strain XL embedded in glycol-methacrylate and oxidized for 5 min with 3% hydrogen peroxide 101

74. Thin section of a cell of *S. faecalis* strain XL after digestion for 45 min with 0.2% pronase 101

75. Thin section of a control cell of *S. faecalis* strain XL embedded in glycol-methacrylate and oxidized for 5 min with 3% hydrogen peroxide 101

76. Thin section of a cell of *S. faecalis* strain XL embedded in glycol-methacrylate and digested for 2 hr with 2500 μg of lysozyme per ml followed by treatment with 0.2% sodium lauryl sulfate for 10 min 101

Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Cores are large, cylindrical structures that have been observed only in group D streptococci. The physiological, chemical, and structural properties of cores were studied in two strains of group D streptococci, *Streptococcus faecalis* ATCC 8043 and *Streptococcus faecalis* strain XL. The ultrastructural characteristics of the cores were studied by the techniques of ultrathin sectioning of chemically fixed bacteria, freeze-etching of fixed and unfixed bacteria, and by negative staining of isolated cores. The morphology of the cores did not vary under these conditions. The cores ranged from 0.1 to 0.22 μm in diameter with internal bores of 0.03 to 0.08 μm and extended the length or width of the cell. The

material of which the cores was composed was amorphous and contained no discernible subunits. Cores which appeared to be forming or disintegrating, however, exhibited a substructure of concentric layers of dense material that resembled unit membrane in thickness and which appeared to be bilayered in some regions.

Cores in *Streptococcus faecalis* strain XL were formed in the cells at a reproducible point in the early stationary growth phase. A period of extensive formation of mesosomal membrane in the cells preceded the formation of cores. Mesosomal membrane decreased in amount starting at the time that cores were first observed. Young cells from logarithmically growing cultures were never observed to contain cores. However, when young cells were (i) permitted to autolyze in 0.01 M potassium phosphate buffer, pH 6.5 at 37 C for 30 min, or (ii) cultured for 30 min in the presence of 1000 units of penicillin G per ml, cores were formed.

The results of cytochemical studies indicated that cores have the following characteristics: (i) they contain protein; (ii) they do not contain any polysaccharide that possesses 1,2-glycol groups; or (iii) any ribonucleic acid detectable by the methods used. Cores are probably not composed of cell wall components as once thought, but the presence of glycerol teichoic acid is not disproved.

Hydrogen ion concentration appeared to be an important factor in the formation of cores. The structures did not form in cells growing in media buffered to remain above pH 6.5, or below pH 5.0 where growth was very slow. Cores disappeared rapidly from cells incubated either at 37 C or at 2 C in buffers or in media that were above pH 6.5. Cores disappeared within 5 min from cells placed in fresh medium at pH 7.2, but they remained stable in fresh medium, or in spent medium filtered from an old culture, if the pH was below 6.5. The formation of cores appeared to be influenced both by growth phase and by pH. Cores disintegrated in spheroplasts and were unstable upon release from the cells, except when they were kept at a low pH or when they were chemically fixed.

Interruption of protein synthesis by the addition of 50 μg of chloramphenical (CAP) per ml to growing cultures resulted in the inhibition of the formation of cores in the cells whether CAP was added 30 min or 2½ hr before the time at which cores were produced normally. This result indicates that cores are not formed from preexisting protein by a self-assembling mechanism. They appear to be assembled either from newly synthesized protein, or to be synthesized by enzymes that are not produced when protein synthesis is interrupted by CAP.

Since cores form after active growth has ceased and after a considerable amount of mesosomal membrane has

been produced in the cells, it is postulated that the structures may represent a labile repository for membranous protein.

INTRODUCTION

The cylindrical cores which occur in group D streptococci are among the largest and most prominent of the structures observed in bacterial cells. The initial electron microscopic studies of thin sections of cores in bacteria were made only in 1968 (11). The work reported in this study contributes to an understanding of the chemical, physiological and ultrastructural nature of these structures. No publications have appeared in the literature concerning any of these properties of cores, and all that is known of them is that they form under conditions where active cell division is inhibited and occur in almost every cell in a culture of group D streptococci (74, and personal observation).

In recent years, ultrastructural techniques have been perfected to the extent that they have contributed much to the understanding of such processes as sporulation, viral infection of cells and photosynthesis. Previously unrecognized and unsuspected structural components of microorganisms are now being observed and studied.

Even structures that could be postulated to exist in bacteria on the basis of physiological data were the objects of controversy until such time as they were

visualized under the electron microscope. A classic example of this was the question of the existence of a cytoplasmic membrane, or plasmalemma, in bacteria. Chapman and Hillier (9) published electronmicrographs of thin sections of gram-positive bacilli in 1953 which represented the best ultrastructural work on bacteria performed by anyone at that time. From the results of their study, the authors erroneously suggested that there was no real membrane in bacteria and that their work supported the contention of Henrici (49) that the cytoplasmic membrane is a surface phenomenon, an optical effect or both. Only four years after this paper was published, however, Chapman and Kroll (10) demonstrated the existence of a "true membrane" in bacteria, reenforced by the later work of Chapman (8) and of Kellenberger and Ryter (67). Another example is the empty "peripheral bodies" also shown in the publication of Chapman and Hillier (9) in 1953 and which are known now to be the often elaborate, membranous structures called "mesosomes."

Evidence has accumulated showing that bacteria possess not only a bilayered plasma membrane but also structures such as mesosomes and extensive intracytoplasmic membrane systems that could represent sites of enzymic activity and compartmentalization of physiological functions. An array of vacuoles, inclusions, membranes, and

tubules of many sizes and shapes have now been observed in bacterial cells (84).

Of the tubular structures reported in bacteria, the core-like structures in group D streptococci are the largest, measuring 0.1 to 0.2 μm in diameter and spanning the breadth or length of a dividing cell. From the results of the limited studies that have been reported (75), it appears that cores occur almost exclusively in group D streptococci. The hollow bore of the core contains ribosomes, so that either part of the cytoplasm is trapped within the cylinder as the structure is formed in the cell, or the core is formed around specific ribosomal configurations (74). Although there has been a number of reports of smaller "microtubular" structures in bacteria (19, 20), the cores in group D streptococci are unique in regard to their large size and the homogeneous material of which they seem to be composed.

The properties of cores that have been studied in this work are: (i) the conditions under which they are formed; (ii) the stage of cultural growth at which they are formed; (iii) their ultrastructural characteristics in chemically fixed and unfixed bacteria; (iv) the factors that govern their stability; (v) their chemical nature; and (vi) the relationship of the formation of these proteinaceous structures to the inhibition of protein synthesis at specific periods of cultural growth.

There are, of course, further questions concerning cores which may be answered at some later time. Such questions as those of the exact role of cores in the synthetic activities of the cell, in particular, their possible relationship to the synthesis of membrane, their quantitative chemical composition, and whether they prevent cell division once they are formed, all require more investigation.

The most intriguing aspect of the formation of cores, however, is that they have been reported in only one group of one genus among bacteria. The elucidation of the chemical specificity responsible for the formation of these large structures will be a significant contribution toward understanding the basis for the presence of these unique structures in the cells of group D streptococci.

LITERATURE REVIEW

The first chance observation of large, tubular structures in group D streptococci was made by Abrams and coworkers in 1964 (1) when they observed these structures in membrane ghosts from protoplasts of *Streptococcus faecalis*. The observation was repeated in 1967 (73), followed the next year by two reports from this same group of workers (11, 74) describing some of the structural characteristics of these large tubules and giving them the name "cores."

Cohen et al. (11) observed barely visible straight lines through the centers of protoplasts of *S. faecalis* under phase contrast microscopy and discovered that they were the stiff core-like structures originally described by Abrams et al. (1). They considered the possibility that the structures were polymeric constituents of systems involved in cell wall synthesis which formed after synthesis was interrupted by growth in penicillin. Looking at thin sections under the electron microscope, they described two types of core structures: (i) a cylindrical core varying in thickness from 0.08 to 0.11 μm with ribosomes in the central bore of the cylinder and which they termed a "nontubular" core; and (ii) an electron

dense "tubular" form from 0.08 to 0.13 μm wide with a tubular wall between 0.01 and 0.03 μm thick and with an irregular arrangement of ribosomes along the structure. The "nontubular" core was observed in cells that still had a compact cytoplasm, whereas the "tubular" cores were observed only in protoplast forms and often could be observed to protrude stiffly outside the protoplast. They observed no confluence with the plasma membrane. From the observations in this paper, the authors assumed that core-like structures were associated only with protoplasts and transitional forms of cells after treatment with penicillin. However, a note added in proof stated that nontubular types of cores had been seen in normal cells cultured in brain-heart infusion broth.

This observation led to the next paper published in 1968 by this group (74) in which they reported on the occurrence of cores in parent strains of several group D streptococci. They found that cores were easy to locate in thin sections of cells from cultures grown for 24 hr and that they ranged in size from 0.10 to 0.16 μm in thickness, with the ends extending near the cytoplasmic membrane. The electron-dense ribosome-like particles in the bore of the core seemed in some preparations to be in a tight helical array. The cores themselves varied somewhat in electron density, and cores fixed with osmium

tetroxide alone were quite indistinct. Cores occurred in 81% of 75 cells examined in 5 sets of serial sections.

These authors suggested two possible explanations of these unusual structures: (i) they represent an accumulation of cell wall precursors following the decrease in the autolytic activity which occurs after the logarithmic growth phase (2, 17); or (ii) the core matrix is composed at least partly of the group D antigen (a glycerol teichoic acid). The latter is present in highest amount during stationary growth (110) and is associated with a ribosomal fraction that can be isolated by centrifugation (133).

A further study of the specificity of core-like structures for group D streptococci was made by McCandless et al. (75) in 1971 in which they readily found cores in cells from 15 of 17 strains of group D streptococci observed after 18, 24, 42, and 48 hours of growth in brain-heart infusion broth. In only one strain did they fail to find cores. In streptococci of Lancefield serologic group A, B, C, E, F, H, K, L, M, and N, in viridans, or in unclassified groups, no cores were observed under any of the conditions studied. The authors were particularly interested in observing cells from strains of group N since this group, like group D, has an intracellular group-specific teichoic acid as antigen. No cores were observed, however.

The only report of core-like structures in a bacterium other than group D streptococci has come from the same group of workers, in 1971 (61). A core-like structure was observed in the stable L-form of *Pseudomonas aeruginosa* B10490, a gram-negative bacterium. The cores were not observed in the parent form of the bacterium.

No work has been reported on the physiology, origin, chemistry, or other characteristics of core-like structures other than two abstracts based on the work presented in this dissertation (S. E. Coleman and A. S. Bleiweis, Bacteriol. Proc., p. 48, 1971; S. E. Coleman and A. S. Bleiweis, Absts. of the Annual Meeting, American Society for Microbiology, p. 76, 1972).

"Microtubular" structures—Many recent reports have been made of small tubular structures in bacteria similar to the microtubules that are observed in eucaryotic cells (69).

While studying the anchorage of flagella in swarmers of *Proteus mirabilis*, van Iterson et al. (126) observed microtubules that were 20 nm in width in thin sections and in negative stains of the bacteria. These were somewhat thinner than most microtubules. Corfield and Smith (19, 20) reported microtubular structures in L-forms of group D streptococci. These appeared to be continuous with the plasmalemma and had an external

diameter of 25 nm and a hollow core 10 to 15 nm in diameter. The tubules were found protruding either into or out of the L-form and were found only in cultures growing in the presence of penicillin.

Some unusual small tubules have been described in *Chondrococcus columnaris* (J. L. Pate and E. J. Ordal, Bacteriol. Proc., p. 32, 1965; M. F. Kuhrt and J. L. Pate, Bacteriol. Proc, p. 165, 1971). These tubules are released when the cells are broken and they measure 28 nm in diameter and vary from 10 to 1,500 nm in length. The most unusual aspect of the structures is that the central cavity widens and narrows from 3 to 30 nm in diameter in a regular pattern. Since large numbers of mesosomes were seen in whole cells, Kuhrt and Pate compared the chemical compositions of the tubules and mesosomal membrane and, finding no significant differences, postulated that the tubules may be derived from the mesosomal membrane.

Rhapidosomes—Perhaps the most extensively studied structures of microtubular form in bacteria are the rhapidosomes. These structures were first observed in 1963 by Lewin (71) in cells of the genus *Saprospira*. Correll and Lewin (21) found that the structures were ribonucleoprotein and that they existed in two forms: (i) a hollow cylinder 33 × 225 nm and (ii) a solid

cylinder of the same dimensions with a "wick-like" appendage. Lewin and Kiethe (72) studied the conditions under which the structures were formed in *Saprospira* and considered that they might represent virus formations due to their ribonucleoprotein composition.

Yamamoto in 1967 (137) investigated the occurrence of rhapidosomes in a variety of species of bacteria and found them in members of the genera *Pseudomonas*, *Photobacterium*, *Proteus*, and *Saprospira*. He did not observe them in *Escherichia*, *Salmonella*, *Shigella*, *Klebsiella*, *Micrococcus*, *Bacillus*, *Mycobacterium*, or *Hydrogenomonas*. The dimensions varied from species to species and the rhapidosomes in cells of *Pseudomonas* did not have the "wick-like" appendage observed in *Saprospira*. Because all cells in cultures of *Pseudomonas* or *Saprospira* appeared to have the structures and since they could be observed under differing conditions of growth and age of culture, Yamamoto considered them to be an integral and functional part of the normal, growing cell. His study also indicated that the structures were embedded in the nucleoplasm of the cells, leading him to postulate that they may have a role analogous to microtubules in eucaryotic cells.

In 1968 Reichle and Lewin (97) were able to separate the two types of rhapidosomes in *Saprospira grandis* by density gradient centrifugation. They obtained

pure preparations of an upper band of forms without wicks and a lower band of forms with wicks.

Cylindrical Ribosomal arrays—Maniloff et al. (76, 77) have reported the observation of rather large, cylindrical arrangements of ribosomes in *Mycoplasma gallisepticum*. These sometimes extended from one side of the cell to the other and were reminescent of the helical arrays noted by McCandless et al. (74) along the bore of nontubular core structures in group D streptococci. Maniloff et al. (76) felt that the cylindrical conformation of the ribosomes might be an artifact occurring in tight packing of the cells but that it was a reflection of ribosomal arrangements in the cell and reflected the symmetry of the ribosomes.

Gas vacuoles and chlorobium vesicles—Cylindrical gas vacuoles are found in many photosynthetic bacteria (6, 88, 112, 129) and halobacteria (60, 68, 117). Cohen-Bazire et al. (13) made a comparative study of the structure of gas vacuoles in blue-green algae, green bacteria, purple sulfur bacteria, and halobacteria. They found that the vacuoles are compound organelles made of many closed, cylindrical gas-containing vesicles with conical ends 80 to 100 nm in width and from 0.2 to 1.0 μm long. The wall of the gas vesicles is a nonunit membrane 2 to 3 nm in thickness with regular striations of a

periodicity of 4 nm oriented at right angles to the long axis of the cylinder. In many blue-green algae the vesicles are arranged in parallel bundles. The gas vacuoles appeared to be homologous organelles in all of these procaryotic groups.

Gas vesicles were isolated by Stoeckenius and Kunau (117) from *Halobacterium* strain Delft by lysing the cells on agar plates with distilled water. The structures remained stable after treatment for 12 hr with 100 μg of pronase per ml of lysate. After lysing the cells, a fraction with a buoyant density of 1.23 g/ml was obtained that consisted of the "intracytoplasmic membrane" or gas vacuoles. The isolated vacuoles were composed of membranes that were 4 nm thick and that consisted mainly of protein. No lipid could be extracted by the use of chloroform-methanol extraction methods.

The gas vacuoles isolated were spindle shaped, occasionally tubular, and had a striated appearance. In freeze-etched cells the striations on the gas vacuoles were highly distinctive. They could be seen on the inner surface of vacuoles in freeze-etched cells and also on the outer surface looking at collapsed vacuoles. Evidence indicates that the vacuole is surrounded by a membrane that can be described as a stack of hoops laterally attached to each other. Disintegrating vacuoles were

observed to break along the striations. The authors found it surprising that no lipid was detectable, although they postulated that it could be covalently bound to the membrane substance. The high buoyant density of 1.23 g/ml made it unlikely that the membrane would have a structure and composition similar to that of other membranes, or that it represents "half of a unit membrane" as suggested by Bowen and Jensen (6). Analysis of amino acids indicated that 70% of the dry weight of the vacuoles was protein and that the proportion of dicarboxylic to basic amino acids was low in comparison with halobacterial envelopes. This finding was in good agreement with the observed resistance of the vacuoles to dissolution in distilled water.

Jost (64) observed gas vacuoles in freeze-etched blue-green algae and reported that the striations were rows of granules on the outer surface. Jost and Matile (65) isolated the gas vacuoles from the blue-green alga *Oscillatoria rubescens* and reported a high lipid and carotenoid content. Stoeckenius and Kunau (117), however, did not feel that this fraction was well characterized since neither the typical shape nor the surface structure could be seen in the purified material.

The only other cylindrical organelle in procaryotic cells that is similar to the gas vacuole is the chlorobium

vesicle which is the site of the photosynthetic pigment system in green bacteria (14, 88). These vesicles have been shown to be completely enclosed by a nonunit membrane (14) which is not connected to or derived from the cytoplasmic membrane.

Tubular spore appendages in clostridia—A number of species of clostridia produce long tubular appendages that are specialized elaborations of the spore coat formed in the cytoplasm of the cell during spore development and which are thought to be formed by self-assembly of subunits (54, 91, 106, 140). In *Clostridium bifermentans* the tubular appendages are about 80 nm in width with spherical subunits arranged in rectangular patterns (140). Some are arranged in a single row of 30 subunits and some in three parallel layers of at least 60 subunits (106).

Isolation and partial chemical characterization of the appendages of *Clostridium taeniosporum* was performed by Yolton et al. (139). Both spore coat and appendages were found to be basically protein and to contain at least 17 common acids. However, the molar ratios of the amino acids varied, and the spore appendages contained 80% dry weight as amino acids whereas the spore coat contained only 68%. The appendages contain glucosamine but not muramic acid and diaminopimelic acid which are common cortical constituents of spores. The appendages contained

glucose and probably rhamnose. Thus, it appears that the spore appendages are largely protein and are differentiated in chemical composition from the spore coat.

Polyhedral bodies and other inclusions—Wildon and Mercer (134) described structures in blue-gree algae that they referred to as "crystalline" bodies. These are now generally called polyhedral bodies (36, 37, 80, 90) and appear to be ubiquitous in blue-green algae (37).

Looking at the ultrastructure of blue-green algae, Gantt and Conti (37) observed long polyhedral bodies, sometimes extending the length of the cell, in *Anacystis nidulans*. These rod-shaped bodies approximate the diameter of the core-like structures in group D streptococci, but they are solid, crystalline structures with regular subunits which have a periodicity and a helical or parallel pitch. No limiting membrane was observed around the polyhedral bodies which showed considerable variation in size and shape.

Interestingly, the authors comment that although polyhedral bodies are ubiquitous in blue-green algae and should be simple to isolate and analyze chemically, this has never been reported.

In addition to the blue-green algae, polyhedral bodies have been reported in *Nitrobacter agilis* (90) and in species of thiobacilli (108). Cells of *Nitrobacter* also contain β-hydroxybutyrate inclusions (111, 121).

Some interesting crystalline inclusions have been reported in bacilli (86), in clostridia (92), in thiobacilli (108), and from phage production in *Escherichia coli* (7). Norris and Proctor (86) reported that *Bacillus thuringiensis* contains protein crystals in the form of parasporal bodies 1 × 0.05 μm in size. This crystal represents one of the toxins produced by the pathogen. Another type of crystalline inclusion was observed to be released from phage-infected cells in the late logarithmic growth phase. These bodies were reported to bear obvious resemblance to the bodies observed by Pope et al. (92) in *Clostridium cochlearium*. It was further suggested by Norris and Proctor (86) that the rod-shaped structures described by Pope et al. (92) could come from overlapping of the lattice planes of the crystal with the hexagonal subunits, which also appear as though they might be hollow cylinders. This second type of crystalline body whose subunits have a center-to-center spacing of 9.5 nm as opposed to the crystals formed from the protein toxin, with a 4.7 by 12.0 nm subunit, appears only in cells infected with phage. This supports the conclusion of Pope et al. (92) that the inclusions observed in clostridia are formed from defective phage production.

Intracytoplasmic membranes—Many species of bacteria contain membrane within the cytoplasm of the cell.

Some possess simple loops of membrane which invaginate from the cytoplasmic membrane and others possess more elaborate structures that sometimes contain layered membrane and which may be independent of the cytoplasmic membrane. These membranous structures can be divided into two general groups: (i) mesosomes, which are whorled invaginations of the cytoplasmic membrane, usually originating at the site of a new septum and considered to be unstable, dynamic structures; and (ii) cytomembranes, which are stable and divide during cell division. Cytomembranes often occur in bacteria with specialized mechanisms for acquiring energy such as the photosynthetic, methane-oxidizing and nitrifying bacteria.

Mesosomes—Investigations into the membranous structures called mesosomes have been in progress for about the last 10 years. The definition of these structures resulted directly from improvements in methods of fixing and embedding bacteria of which the classical work of Ryter et al. in 1958 (102) contributed the most. Membranous structures have been seen in many genera of gram-positive bacteria including *Mycobacterium* (62), *Bacillus* (125), *Streptomyces* (43), *Staphylococcus* (3), *Lactobacillus* (J. P. Brown, Bacteriol. Proc., p. 44, 1969), and *Micrococcus* (105). These structures have been called "mesosomes" by Fitz-James (33), "plasmalemmasomes" (29), and "chondrioids" (125). The term mesosome is now generally accepted.

Mesosomes have inspired much speculation concerning their possible functions within the cell. Their general morphology and membranous origin suggested to many workers that they might have a function similar to mitochondria (34, 43, 127). The latter suggestion was supported by the work of Vanderwinkel and Murray (123) showing the cytochemical localization of the reduction of triphenyltetrazolium chloride in mesosomes in bacilli. Their presence in the nuclear region raised speculation that they might be involved in nuclear division and function (41, 100, 125). The observation by Wolstenholme et al. (136) that the number of mesosomes increases significantly in competent cells of *Bacillus subtilis* during transformation has also advanced the idea that these structures play a role in the process of transformation, or that cells take up deoxyribonucleic acid at the same time that mesosomes form.

Mesosomes have been postulated to be involved in cell wall synthesis since the first observations by Chapman and Hillier (9) of "peripheral bodies" located at the forming edge of the transverse cell wall. In three-dimensional reconstructions of cells of *B. subtilis* (101), all nascent septa were observed to be in contact with mesosomes. It was observed further that mesosomes were lost during the formation of protoplasts (38, 103) and during autolysis of group D streptococci (personal observation).

Imaeda and Ogura (62) found an intimate relationship between the formation of mesosomal systems and cell division in mycobacteria and observed a moderately dense substance in the membrane system which they postulated to be cell wall precursor material.

A number of workers have isolated mesosomes from species of *Bacillus* (31, 35, 95), and from *Staphylococcus aureus* (93, 119). Reaveley and Rogers (96) and Ferrandes et al. (31) found that NADH oxidase and succinate dehydrogenase activities were very low or absent from mesosomal membranes, whereas they were about 30 times higher in the cytoplasmic membranes, thus indicating that mesosomal membranes are differentiated in function from the cytoplasmic membrane.

Cytomembranes—Some of the most elaborate systems of cytomembranes are found in the autotrophic nitrifying bacteria. Murray and Watson in 1965 (85) looked at the structure of *Nitrosocystis oceanus* and *Nitrosomonas europaea* which produce energy by oxidizing ammonia to nitrite and at *Nitrobacter agilis* which performs the more complicated oxidation of nitrite to nitrate for energy production. They discovered that *Nitrosocystis oceanus* had a central "membranous organelle" which appeared to be independent of the plasma membrane although rare intrusions from the plasma membrane were observed. The organelle consisted of around 20 vesicles flattened to form layers

of membrane 10 nm thick which stretched parallel to the short axis of the cell. The outer surfaces were in contact and formed a triplet structure with an accentuated center line. The lamellae almost transversed the cells, displacing the cytoplasm and nucleoplasm, and the membranous organelle divided at the same time as the cell, appearing to be a constant, permanent part of the cellular structure. In *Nitrosomonas europaea* the cells contained paired lamellae around the periphery of the cell which did not fuse or form regular associations. The authors considered them to be equivalent to the vesicles in *Nitrosocystis* but they were parallel and close to the plasma membrane. Cells of *Nitrobacter agilis* were observed to have a unique plasma membrane with a dense layer 5 nm thick on the inside of the membrane that was preserved in the parallel lamellae which formed around the periphery of the cell.

Murray and Watson (85) felt that the systems of cytomembranes in these organisms must be related to their specialized mechanisms for energy production and postulated that the regular spacing of the membranes in *Nitrobacter agilis* might be related to the more complex oxidation of nitrite to nitrate that is carried out by bacteria of this genus. The formation of closely packed fusing lamellae in cells has counterparts in the formation of grana in chloroplasts (79) and in the stacked lamellae found in retinal rods (27).

Further studies of the cytomembrane system of *Nitrosocystis oceanus* were performed by Remsen et al. (98) in which they compared thin sectioned, negatively stained, and freeze-etched preparations of this species. In freeze-etched cells the membranes were covered with particles from 8 to 12 nm in diameter. When disrupted and differentially centrifuged, membrane fragments observed by negative staining from the pellet centrifuged at 2,000 × g showed stalked particles of 7 to 8 nm and those from the pellet after centrifugation at 39,000 × g exhibited a crystalline array of subunits with a periodicity of 3 to 4 nm. In chemically fixed cells particles of 4 nm were found embedded in the matrix of the membranes. The results suggested to them that the larger particles of 8 to 12 nm were probably enzyme complexes whereas the smaller particles of 3 to 4 nm represented a structural protein, perhaps a lipoprotein from the membrane. The authors concluded from this structural study that the cytomembranes of bacteria must be less specialized than mitochondrial membranes. Bachmann et al. (2) showed that cristae membrane contains four components of the electron transport chain as well as enzymes such as adenosine triphosphatase. The outer membrane contains other mitochondrial enzyme systems such as those that implement such processes as citric acid cycle oxidation and fatty acid oxidation.

Since no comparable outer mitochondrial membranes exist in bacteria, Remsen et al. (98) concluded that all the enzymes present must occur together on the membrane. Their work led them to speculate further that the cytomembranes of *Nitrosocystis oceanus* may have been derived from plasma membrane but evolved into highly specialized and permanent organelles that perhaps represent an intermediate step in evolution between simple bacterial membranes such as mesosomes and the more specialized membranes of mitochondria and chloroplasts.

Looking at the physiological factors that may control cytomembrane formation in nitrifying bacteria, Pope et al. (90) cultured cells of *Nitrobacter agilis* autotrophically and heterotrophically in the absence of nitrite. They observed that the morphology of the cytomembranes were similar under both conditions, but the lamellar membrane system was less prominent in heterotrophically grown cells than in autotrophically cultured cells. Observation by van Gool et al. (124) on frozen-etched cells of *Nitrobacter*, confirmed the earlier picture of the ultrastructure of the membranes obtained by studies of thin sections of the cells.

Methane-utilizing bacteria and marine bacteria— Quite recently, ultrastructural studies have been made of the extensive cytomembrane systems found in the

methane-oxidizing bacteria (24, 94, 113, 114). Smith and Ribbons (113) showed that extensive peripheral cytomembranes occurred in *Methanomonas methanooxidans* which consisted of triple-layered unit membranes that appeared to be fused in pairs and to be regularly spaced. It was not possible to see any continuity between the cell membrane and the intracytoplasmic membranes. In yet another genus of methane-oxidizing bacterium, *Methylococcus capsulatus*, Smith et al. (114) showed the presence of very regular arrays of disc-shaped stacks of cytomembranes formed throughout the cells. In one electronmicrograph a stack of 27 discs could be observed extending through the center of a dividing cell. These membranes differ from those previously observed in nitrifying bacteria, but like the latter, are thought to be the site of activity for the specialized mechanism that permits the oxidation of methane for production of energy.

In marine bacteria, interesting myelin-like membranous structures have been observed by Felter et al. (30) in cells of *Vibrio marinus*. The structures occurred more frequently and were more complex in late logarithmic and stationary phase and were either myelin-like or consisted of membranes randomly arranged throughout the cells. The authors proposed the name "myelemma" for the myelin-like formations. These structures were not observed to be

connected with the plasma membrane, although they were often observed in approximation to membrane or associated with vacuoles and circular membrane. Similar random membrane structures have been observed in marine species of luminescent bacteria belonging to the genus *Photobacterium* (M. E. Byrd, personal communication). These structures were often observed to be distal from the cytoplasmic membrane and to contain dense-staining material of unknown origin.

Photosynthetic bacteria—In the Thiorhodaceae and the Athiorhodaceae the photosynthetic apparatus appears to be a membranous extension of the cytoplasmic membrane. In the Chlorobacteriaceae the photosynthetic apparatus consists of a series of membrane-bound vesicles and in the Thiorhodaceae there is a range of variation from membranous vesicles to tubular membranes and lamellar systems of membranes (28). Members of the Athiorhodaceae have been most widely studied and the photosynthetic membranes have been observed to range from membranous vesicles to elaborate lamellar systems.

In the purple bacteria the most common membranous structure is the vesicular type which occurs in most species examined thus far (5, 12, 56, 57, 58). Certain purple bacteria contain internal membranes that form multilayered lamellae and these include most species of *Rhodospirillum* (42) and *Ectothiorhodospira mobilis* (59, 122).

The green bacteria have a completely different photopigment system from the purple bacteria (14). The Chlorobacteriaceae are the only group of photosynthetic organisms in which the photopigment system is not associated with membranes but is located in specialized organelles called chlorobium vesicles (14, 55). For this reason, the photosynthetic apparatus of green bacteria is more complex and specialized than that of purple bacteria or of blue-green algae and can be regarded, perhaps, as the most highly differentiated of procaryotic organisms (14).

Ultrastructurally, the membrane systems of photosynthetic bacteria have been reported to change in relation to light intensity and other environmental factors. Holt and Marr (56, 57, 58) reported that cells of *Rhodospirillum rubrum* grown at low light intensity contained more internal membrane than cells grown at high light intensity. This indicated that the regulation of chlorophyll content of the cell depended upon the amount of membrane formed and that the membrane contained a constant amount of chlorophyll. Gibbs et al. (40) found that the number of internal membranes varied with the amount of bacteriochlorophyll resulting from varied light intensity and that with small amounts of bacteriochlorophyll the membranous invaginations were tubular, but with larger amounts they were disc-shaped and from 2 to 10 discs occurred in a stack.

However, in working with the green bacterium *Chloropseudomonas ethylicum*, Holt et al. (55) found that the specific chlorophyll content of the isolated vesicles varied in relation to light intensity over a wide range and that the regulation of chlorophyll content was achieved both by a change in the number and in the specific chlorophyll content of vesicles in the cells.

Conti and Hirsch (18) looked at the membrane systems of the unusual budding bacteria *Rhodomicrobium* and *Hyphomicrobium*. The membranes of these bacteria were continuous with the cytoplasmic membrane and were identical in morphology. *R. vannielii* and *H. vulgare* are the sole representatives of their genera and they multiply by forming daughter cells at the end of a hypha. *H. vulgare* and related strains are aerobic organotrophs while *R. vannielii* is an anaerobic photoorganotroph. However, it was discovered by Conti and Hirsch that morphologically they are almost identical. This supports the suggestion made by Van Niel (128) that *H. vulgare* is the colorless counterpart of *R. vannielli*. This work also agrees with the observations of Boatman and Douglas (5) that these species have peripheral lamellar membrane systems and intracytoplasmic membranes, both 9 nm wide, which in some areas appear to be paired.

From the work that has been performed on ultrastructural changes in the three groups of

photosynthetic bacteria, it seems apparent that these organisms vary in their chlorophyll-membrane relationships and that these relationships are markedly affected by environmental changes.

These types of membrane interrelationships lend themselves most efficaciously to study by the technique of freeze-etching. At this time, only one photosynthetic bacterium, the marine species, *Ectothiorhodospira mobilis*, belonging to the Thiorhodaceae, has been investigated by freeze-etching (59).

From this review of the literature, it appears rather clear that most ultrastructural components of microorganisms that are now recognized were observed only in the very recent past as techniques became adequate to permit this. It is also apparent that the nature and function of most of the structures observed in procaryotic cells are still poorly understood. Little more than the general structure and only a limited knowledge of the occurrence and function of most structures is known. This includes the core-like structures observed in group D streptococci.

MATERIALS AND METHODS

Cultures—Two stains of group D streptococci were used in these studies, *Streptococcus faecalis* strain XL isolated from oral sources and *Streptococcus faecalis* ATCC 8043.

Media—Most experiments were performed by culturing the bacteria in Todd-Hewitt broth (Difco) adjusted to a final pH of 7.2. Other experiments involved the use of brain-heart infusion broth (Difco) and trypticase soy broth (Baltimore Biological Laboratories, Baltimore, Maryland). Where Todd-Hewitt agar was used, 2% agar (Difco) was added to the broth.

Balanced cultural growth—Cultures used for studies of the formation of cores were grown under conditions in which optical density and dry weight were increasing at the same rate, and the lag phase was eliminated.

To achieve these conditions of growth (E. P. Previc, personal communication), initial inocula were prepared by spreading 0.1 ml of an overnight broth culture on a plate of Todd-Hewitt agar, incubating the plate at 37 C overnight and preparing an inoculum for the first flask of 100 ml of broth by washing growth from the plate with sterile broth. The flask was incubated at 37 C with gentle stirring over an

insulated magnetic stirrer. When the culture reached mid-logarithmic growth, 10 ml were transferred from the first flask into a second flask of 100 ml of broth which had been prewarmed to 37 C, and the same sequence repeated with a transfer of 30 ml into a third flask of 300 ml of broth.

In order to ascertain when the lag phase of growth had been eliminated, the optical density of the culture was measured at 660 nm in a Gilford spectrophotometer, and the dry weights of samples of cells from the culture were measured at intervals of 30 min for the first 4 hr of growth.

Measurement of the dry weights of samples after 30 min and 1 hr of growth was made by taking samples of 100 ml of culture using a 50 ml volumetric pipette and filtering the samples through a nuclepore filter (General Electric Co.) with a pore size of 0.5 μm. All other samples consisted of 50 ml of culture. A control sample of 50 ml of sterile broth was filtered, and the weight of this filter was subtracted from the weight of the other samples.

The samples were filtered with a vacuum produced by the pressure of running water. The filtration time for the samples was 1 min 45 sec ± 15 sec. The filters containing the samples were immediately placed in an oven

set at 60 C and dried for several minutes until the filters curled. After a cooling period of 2 min, the filters were weighed on a Mettler balance, reheated, cooled, and reweighed until the weight stabilized.

The dry weights were determined for each sample by subtracting the average initial weight of the filter paper from the average final weight of the filter paper containing the sample minus the weight of the filter paper through which sterile medium had been filtered.

Cytochemical Studies—Staining for the presence of polysaccharides containing 1,2-glycol groups in thin sections of bacteria was accomplished by use of the silver methenamine staining procedure. The method used was essentially that of de Martino and Zamboni (25) as based on the earlier work of Gomori (44) and Jones (63) and incorporating modifications suggested by Thiéry (120), Walker and Short (131), and Petitprez and Derieux (87).

Bacteria to be used in this procedure were fixed for 30 min in the cold with 2% glutaraldehyde (Polysciences, Warrington, Pennsylvania), in 0.09 M sodium cacodylate buffer, pH 7.2, washed twice with the buffer and fixed for 10 hr with 1% osmium tetroxide (OsO_4, Engelhard Industries, Newark, New Jersey) in 0.1 M veronal buffer with tryptone (101) washed, dehydrated, and imbedded in an epon-araldite plastic mixture (81).

Thin sections (60 to 80 nm) of the bacteria were placed on stainless steel grids with a size of 200 mesh for the staining procedure. The stock staining solution consisted of: 100 ml of 3% hexamethylenetetramine (Eastman Organic Chemicals, Rochester, New York) in bidistilled water and 5 ml of 5% silver nitrate in bidistilled water. The staining solution was prepared by mixing 10 ml of the stock solution with 10 ml of bidistilled water and 0.8 ml of 5% borax (sodium borate). The staining solution was prepared just before use, centrifuged for 30 min at 2,000 × g and kept in the dark.

In order to perform the staining procedure, the stainless steel grids containing the sectioned bacteria were placed in small plastic containers and oxidized with 1% periodic acid for 5 min at room temperature. They were washed with distilled water, dipped into 5% sodium bicarbonate as an aid in stopping the oxidation, again washed and placed in the staining solution in the dark for 20 min in a water bath set at 60 C.

The grids were washed three times in bidistilled water and observed without poststaining under the electron microscope. The following controls were used: (i) untreated sections poststained for 15 min with 0.5% uranyl acetate and for 5 min with lead citrate (99); (ii) sections oxidized for 5 min with 1% periodic acid and poststained

as above; and (iii) unoxidized sections stained with the staining solution.

Digestion of cores by enzymes—Bacteria to be sectioned and digested by enzymes were fixed with 2% glutaraldehyde in the cold and embedded in either a mixture of epon-araldite plastic (81) or in the water-soluble glycol-methacrylate (70). The thin sections (60-90 nm) were placed on acid-cleaned stainless steel grids and oxidized for 1 min with 3% hydrogen peroxide.

The methods utilized in digesting the sections with enzymes were those of Walker (130), Granboulan and Leduc (45), Monneron and Bernhard (82), Weintraub et al. (132).

The enzymes were purchased from Sigma Chemical Co., St. Louis, Missouri, and were as follows: trypsin (bovine pancreas, 2× crystallized, 9800 BAEE units/mg); deoxyribonuclease (beef pancreas, 720 Kunitz units/mg); ribonuclease-A (bovine pancreas 6× crystallized, protease-free Type 1-A, 90 Kunitz units/mg); lysozyme (egg white, Grade 1 3× crystallized, 25,000 units/mg); pronase (Type VI, 3-4 units/mg); and lipase (Type VI, from hog pancreas, 100 units/mg). The enzymes, concentrations, and the periods of time that digestion of the sections was allowed to proceed at 37 C are described in Table 1.

Autolysis of streptococci—The autolysis of young cells from logarithmically growing cultures was induced by

Table 1. *Conditions for the Enzymic Digestion of Cores from Thin Sections of Streptococcus faecalis Strain XL*

Enzyme	Concentration	Diluent	pH	Time of Digestion at 37 C
Pronase	0.2%	0.2 M Tris-HCl buffer	7.4	10, 20, 30, and 45 min
Ribonuclease	0.1%	Distilled water, adjusted with NaOH	6.8	30 min, 2, and 6 hr
Deoxyribonuclease	0.1%	Distilled water, adjusted with NaOH	7.4	6, 12, and 24 hr
Lysozyme	25,000 μg/ml	0.01 M Tris-HCl buffer	8.3	30 min, 1, 2, and 6 hr[a]
Trypsin	0.5%	0.05 M Tris-HCl buffer + 0.01 M $CaCl_2$	8.0	30 min, 1, 2, and 6 hr
Lipase	0.1%	0.05 M Tris-HCl buffer + 0.01 M $CaCl_2$	8.0	30 min, 1, 2, and 6 hr

[a]Grids then floated on 2% sodium lauryl sulfate for 10 min and washed with distilled water.

the procedure of Shockman and Martin (109). The cells were harvested by centrifugation, washed three times with distilled water and allowed to autolyze in 0.01 M potassium phosphate buffer, pH 6.5 at 37 C for periods of 15, 30, and 60 min.

Induction of cores by penicillin—The formation of cores in cells from logarithmically growing cultures was induced by the addition of 1000 units of potassium penicillin G (Eli Lilly and Co., Indianapolis, Indiana) per ml. Samples of the bacteria were taken after 5, 10, 20, and 45 min incubation with the antibiotic, fixed and embedded for observation under the electron microscope (see below).

Formation of spheroplasts—Spheroplasts of cells containing cores were made by the method of Coleman et al. (15, 16). The cultures were incubated until they reached stationary growth, and the cells were harvested by centrifugation, washed three times with distilled water, and incubated for 30 min at 37 C with 250 μg of lysozyme per ml.

Effect of pH on stability of cores—In order to observe the influence of pH on the stability of the core structure in both cells and spheroplasts, the organisms were incubated in buffer systems at varying pH values for periods of 30 and 60 min at 37 C and in the cold at 2 C.

The following buffers were used with and without 0.5 M sucrose: 0.1 M HCl-KCl buffer, pH 2.0; 0.1 M citrate buffer, pH 4.0; 0.1 M potassium phosphate buffer, pH 7.0; 0.1 M Tris buffer, pH 7.2; 0.1 M potassium phosphate buffer, pH 8.0; and 0.1 M Tris buffer, pH 8.4.

In other studies where cells were incubated in the growth medium and the influence of alteration in the pH was observed, the pH of the medium was raised or lowered by the addition of 1 N NAOH or 1 N HCl. In experiments in which cultures were permitted to grow in a buffered medium, Todd-Hewitt broth was prepared in 0.02 M potassium phosphate buffer, pH 7.2.

Isolation of cores—Numerous methods were tested for lysing cells for the recovery of intact core structures. Lysis by lysozyme was accomplished by suspending the cells in 250 or 2500 μg of lysozyme per ml in distilled water at pH 6.0 at 37 C for periods of 5 to 30 min (15). Lysis of the lysozyme-treated cells was then effected by washing the cells in distilled water and adding cations in the form of 0.2 M NaCl, 0.2 M $MgCl_2$ or a mixture of the two. In some cases, lysis was effected by the addition of the cationic detergent sodium lauryl sulfate at a concentration of 0.2%.

A modification of the method of Schaechter et al. (107) was also used to lyse the streptococci. In this

procedure, the cells were incubated in 0.5 M sucrose for 2 to 5 min with 250 μg of lysozyme per ml at 37 C and then poured into frozen, crushed 0.5 M sucrose which rapidly lowered the temperature of the suspension of cells to 0 C in order to effect lysis gently in the cold.

Mechanical lysis of the cells for the release of cores was performed in a Braun homogenizer (Will Co., Rochester, New York) with 1 part ballotini glass beads (0.17 mm) to 2 parts of cell suspension. The cells were broken for periods of 45 sec to 3 min in the cold.

Differential centrifugation was used to obtain a crude isolation of the cores. The cell lysate from the Braun homogenizer was centrifuged in the cold at 2000 × g for 5 min and the resulting supernatant fluid was then centrifuged at 20,000 × g for 60 min. The pellet from the second centrifugation contained the core structures.

In some instances the cells were fixed with 2% glutaraldehyde in the cold for 30 min to 2 hr before breakage in the Braun homogenizer.

Stabilization of core structures—The methods tested for the stabilization of cores for isolation were: (i) treatment with 30% ethanol in the cold for 30 min before breakage of cells; (ii) heating of cell suspension at 80 C for 20 min; (iii) addition of 12% hexylene glycol to lysed cells (116); and (iv) breakage of the cells in

the solution used by Dirksen (26) and Mazia and Dan (78) for the stabilization of isolated microtubules from sea urchin eggs (1 M sucrose, 10^{-3} M ethylenediamine-tetraacetate (EDTA) and 0.15 M dithiodiglycol or dithiodipropanol).

Inhibition of protein synthesis—Protein synthesis was inhibited in growing cultures by the addition of 50 μg of chloramphenicol (Sigma) (138) per ml at a period of 1-1/2 hr before the initiation formation of cores in the culture or at 30 min before initiation.

Electron microscopy—Samples of bacteria were prepared for observation under the electron microscope by collecting them by filtration on a millipore filter with a 0.45 μm pore size and chemically fixing them by one of the following methods: (i) the technique of Ryter and Kellenberger (102) in which the cells are prefixed in the culture medium with 0.01% osmium tetroxide (OsO_4) for 5 min, followed by overnight fixation with 1% OsO_4 in 0.1 M veronal buffer, pH 6.1, followed by postfixation with 2% uranyl acetate in veronal buffer for 2 hr; (ii) fixation by 2% glutaraldehyde in the cold for periods of 15 min to 2 hr in the presence of 0.1 M sodium cacodylate buffer, pH 7.2; and (iii) fixation by 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 in the cold for 30 min to 2 hr followed by fixation with 1%

OsO_4 in 0.1 M veronal buffer, pH 6.1 for 1 to 10 hr at room temperature or in the cold overnight; or (iv) prefixation of the cells in the culture medium with 0.01% OsO_4 for 5 min followed by fixation with 2% glutaraldehyde in the cold for 30 min to 2 hr in 0.1 M cacodylate buffer, pH 7.2 followed by fixation with 1% OsO_4 for 1 to 6 hr at room temperature or overnight in the cold in 0.1 M veronal buffer, pH 6.1 with postfixation for 30 min to 1 hr with 2% uranyl acetate in veronal buffer, pH 6.1. In all cases 0.01% tryptone broth or Todd-Hewitt broth were added to the samples during fixation. The amino acids in the broth aids fixation of ribosomes (102).

After fixation the samples were embedded in 2% agar (Ionagar, Colab Laboratories, Chicago Heights, Illinois), cut into blocks 1 mm square and dehydrated through a series of 25, 50, 75, 95, and 100% ethanol and placed in acetone or propylene oxide for embedding in plastic. Three different plastics were used for embedding the bacteria. The method used most frequently was that of Mollenhauer (81) in which a mixture of epon 812 and araldite 506 was made into a stock solution (stock number 2; 62 ml of epon 812, 81 ml araldite 506, 4-8 ml dibutyl phthalate and mixed 3 parts stock with 2 parts of dodecenylsuccinic anhydride (DDSA) with the addition of the accelerator DMP-30 in an amount to give the desired hardness of plastic.

The more rapid embedding procedure of Coulter (22) was also used in which DDSA and araldite 502 were mixed in a ratio of 0.9:1.0 with the addition of 1.5% DMP-30.

For digestions of thin sections by enzymes bacteria were embedded in a water-soluble plastic (70). In this procedure an embedding mixture was prepared by mixing 7 parts of 97% glycol-methacrylate (GMA) and 3% distilled water plus 3 parts of nondestabilized butyl methacrylate to which 2% Luperco was added. The embedding mixture used for impregnation was partially polymerized by placing a small amount of the embedding mixture in a large container that could be heated. A thin layer of the plastic was heated over a bunsen burner with rapid swirling until boiling began in about a minute and then the container was plunged rapidly into a large bath of ice water.

The agar blocks of fixed bacteria were embedded in the prepolymer by dehydration in: (i) 80% GMA monomer plus 20% distilled water for 20 min; (ii) 97% GMA monomer plus 3% distilled water for 20 min; and, finally, (iii) unpre-polymerized embedding mixture for 20 min followed by impregnation in the prepolymer of the embedding mixture for an overnight period.

For embedding, the blocks were placed in fresh prepolymer in gelatin capsules and polymerized by UV light

of long-wavelength in the cold for 1 to 3 days until the plastic was hard.

Sectioning of the plastic-embedded material was performed with a diamond knife (DuPont de Nemours) on a Porter-Blum MT-2 ultra-microtome (Sorvall, Inc., Norwalk, Connecticut). The ultra thin sections were poststained for 15 min with 0.5% uranyl acetate and for 5 min with lead citrate (99).

The sections were observed on an Hitachi HU11-E electron microscope (Hitachi, Ltd., Tokyo, Japan), at 75 or 100 KV and photographs were made on DuPont Cronar film. The grids were sometimes carbon-coated in a Balzers vacuum evaporator (Balzers, Fürstentum, Liechtenstein).

Negative staining—Negative stains were prepared with 2% phosphotungstic acid, pH 6.0, or with 2% aqueous uranyl acetate. The material was spread on the surface of grids covered with a thin layer of 0.25% formvar plastic, permitted to partially dry and then the negative stain (with 0.01% bovine serum albumin to aid spreading) was added and the stain subsequently drawn off with filter paper. The grids were observed under the electron microscope within a day after preparation.

Freeze-etching—The freeze-etching (or freeze-fracturing) of samples of both fixed and unfixed bacteria was performed on a Balzers BA360M freeze-etch apparatus

Balzers Co., Fürstentum, Liechtenstein) using the methods of Moor and Mühlethaler (83) and Steere (115).

In this procedure, a small drop of sample in 30% glycerol or distilled water was placed on a gold or copper disk measuring 3 mm in diameter and frozen at -150 C in Freon 22. The sample was transferred to liquid nitrogen and stored until used.

The freeze-etching procedure was accomplished by placing a disk containing a frozen specimen on a stage cooled to -150 C on the Balzers apparatus. The specimen was then placed in a bell jar under a vacuum of 10^{-5} Torr or better and the temperature of the stage raised to -100 C. The fracturing of the sample involved passing a cold knife blade through the frozen drop of material until a fracture face was formed. The sample was permitted to etch (sublimate) for 1 to 4 min. The replica of the fracture face of the cells was then formed by first shadowing the fractured cells with platinum and then evaporating carbon to form a carbon replica.

Cellular material was cleaned from the replicas by placing them in 50% chlorox overnight, followed by washing for 1 hr in distilled water. The replicas were picked up on copper grids and observed under the electron microscope.

RESULTS

Structure of cores in ultrathin-sections and in freeze-etched cells—A comparison was made of the effects of various chemical fixatives on the morphology of cores in ultrathin sections of bacteria. The findings are summarized in Table 2. Fixation in 2% glutaraldehyde in the cold from 15 min to 2 hr gave good fixation of the core-like structures. Glutaraldehyde is perhaps the best cross-linking reagent known for proteins (48). Cores in cells of *Streptococcus faecalis* strain XL fixed for 30 min in 2% glutaraldehyde and 3 hr in osmium tetroxide (OsO_4) (Fig. 1) are the same morphologically as freeze-etched, unfixed bacteria (Figs. 2 and 3) or as a cell fixed with 2% glutaraldehyde for 30 min before freeze-etching (Fig. 4). A comparison of cores in thin sections and freeze-etched preparations of *S. faecalis* ATCC 8034 (Figs. 5, 6, 7, and 8) shows their morphology to be the same.

Occasionally a fracture revealed the outer side of the cylindrical core (Fig. 9). No evidence of structural subunits was seen in any of these preparations. Looking at a fracture through the cytoplasmic membrane of a cell

Table 2. *Comparison of the Effects of Chemical Fixation on the Morphology of Cores in Group D Streptococci*

Fixative	Nature of fixation (48)	Appearance of cores and other structures
Glutaraldehyde[a]	Cross-links protein, does not fix membrane, shrinks cell, preserves fine structure well	Good fixation of cores, ribosomes, cell wall, does not fix mesosomes or nuclear material
Osmium tetroxide[b]	Fixes lipid, membranes, does not fix protein well, causes swelling of cells	Cores are indistinct and difficult to see in cells. Mesosomes and cell wall are fixed well
Sequential fixation: (1) glutaraldehyde, (2) osmium tetroxide[c]	Fixes lipid and protein	Good fixation of cores, ribosomes, cell wall, does not fix mesosomes
Sequential fixation: (1) osmium tetroxide, (2) glutaraldehyde, (3) osmium tetroxide,	Fixes lipids, protein and nuclear material	Cores, ribosomes, cell wall, nuclear material, and mesosomes are fixed well

Table 2—*Continued*

Fixative	Nature of fixation (48)	Appearance of cores and other structures
(4) uranyl acetate[d]		

[a]Fixation with 2% glutaraldehyde in the cold from 15 min to 2 hr.

[b]Fixation by the technique of Ryter and Kellenberger (102).

[c]Fixation by 2% glutaraldehyde in the cold for 15 min to 2 hr followed by fixation at room temperature by 1% osmium tetroxide for 1 to 6 hr.

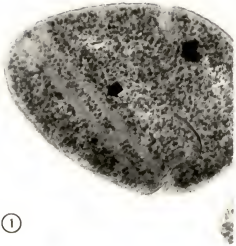
[d]Prefixation with 0.01% osmium tetroxide in the medium for 5 min, then fixation by 2% glutaraldehyde in the cold for 30 min followed by 1% osmium tetroxide for 1 to 3 hr and 2% uranyl acetate at room temperature for 30 min.

Fig. 1. Longitudinal section of a core in *Streptococcus faecalis* strain XL fixed for 30 min in 2% glutaraldehyde in the cold and for 3 hr in 1% osmium tetroxide at room temperature. (×60,000)

Fig. 2. Freeze-etched, unfixed cell of *S. faecalis* strain XL after growth for 12 hr in Todd-Hewitt broth at 37 C. (×83,000)

Fig. 3. Freeze-etched, unfixed cell of *S. faecalis* strain XL after growth for 12 hr in Todd-Hewitt broth at 37 C. (×48,400)

Fig. 4. Freeze-etched cell of *S. faecalis* strain XL fixed for 2 hr in 2% glutaraldehyde in the cold. The morphology is the same as in the unfixed, freeze-etched cells. (×81,400)



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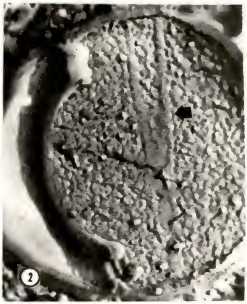

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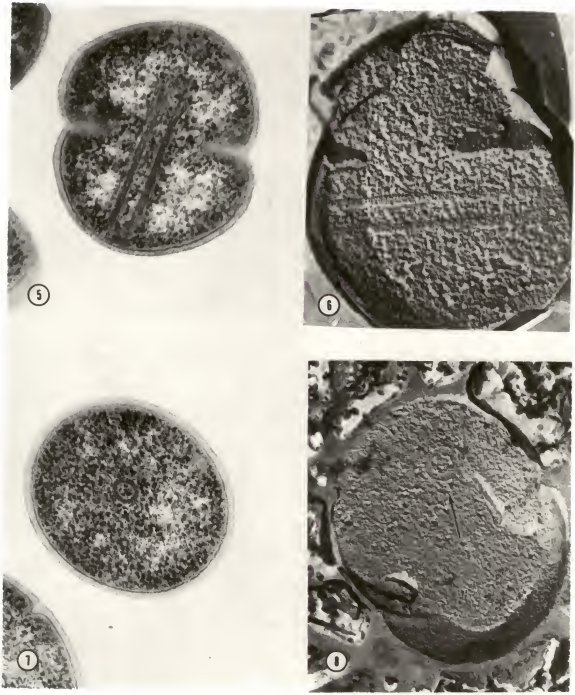
Fig. 5. Thin section of *S. faecalis* ATCC 8043 fixed for 30 min with 2% glutaraldehyde in the cold and for 3 hr in 1% OsO_4 at room temperature. The dense core is apparent through the center of the cell. (×50,300)

Fig. 6. Freeze-etched, unfixed cell of *S. faecalis* ATCC 8043 showing a longitudinal fracture through a core. (×75,700)

Fig. 7. Cross section of a core in a thin section of *S. faecalis* ATCC 8043 fixed for 30 min with 2% glutaraldehyde in the cold and for 3 hr in 1% OsO_4 at room temperature. (×61,600)

Fig. 8. Freeze-etched, unfixed cell of *S. faecalis* ATCC 8043 showing a cross section of a core. (×62,600)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of *S. faecalis* strain XL taken from the stationary growth phase (Fig. 10) distinctive round areas were seen that corresponded in size and shape to that of a cross section of the core structure. These configurations occurred frequently in cells taken from cultures at a stage where they were known to form cores but were not seen on fractures of membranes of young cells that did not contain cores.

There was a distinct difference in the preservation of the core under various methods of fixation. In cells fixed with OsO_4 according to the method of Ryter and Kellenberger (102) (Fig. 11) the structure of the core was indistinct and apparently poorly preserved. While OsO_4 is an excellent fixative for lipids, it is less effective as a cross-linking agent for protein than is glutaraldehyde (48). There is excellent preservation of membrane and nuclear material, however. Cores are fixed well by glutaraldehyde alone (Fig. 12). The cell shown in this figure was fixed for 30 min in the cold by 2% glutaraldehyde after being cultured for 24 hr at 26 C. The cores are well-defined and prominent in cultures incubated at lower temperatures.

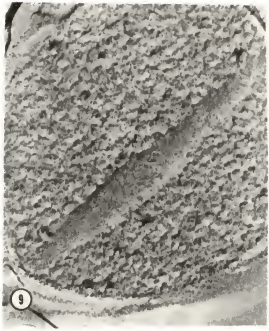
Observations of isolated core structures—Cores were isolated from the cells of *S. faecalis* strain XL by rupturing the cells in a Braun homogenizer under conditions in which they were stable and by crudely separating them

Fig. 9. Freeze-etched, unfixed cell of *S. faecalis* strain XL showing the surface of a core. (×85,500)

Fig. 10. Fracture through the membrane of an unfixed, freeze-etched cell of *S. faecalis* strain XL showing a round configuration (arrow) thought to represent the end of a core. (×44,200)

Fig. 11. Thin section of a cell of *S. faecalis* strain XL fixed by the Ryter-Kellenberger technique (102) with 1% OsO_4 for 16 hr at room temperature. The structure of the core is not distinct in cells fixed by this method. (×53,700)

Fig. 12. Thin section of a cell of *S. faecalis* strain XL cultured for 24 hr at 26 C and fixed for 30 min with 2% glutaraldehyde in the cold. (×68,300)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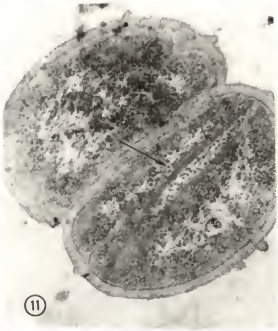
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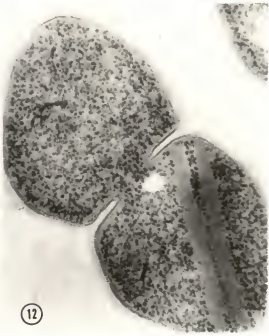
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from the cellular lysate by differential centrifugation. Cores isolated from cells that had been fixed for 30 min with 4% glutaraldehyde and then disintegrated in the Braun homogenizer were observed by negative staining with 2% phosphotungstic acid at pH 6.0 (Figs. 13 and 14). The rod-like structures obtained were uniform in diameter and were not observed in lysates from young cells that did not contain cores. Cores released from cells disrupted in the presence of 0.1 N HCl-KCl buffer, pH 2.0 (Fig. 15) remained stable at this low pH. Apparently the ribosomes remain in the bore of the cylindrical core so that the negative stain does not penetrate into the structure.

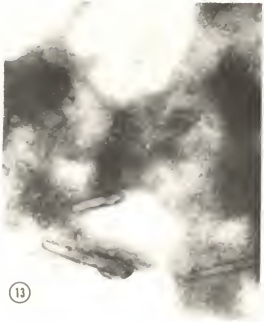
Dimensions of cores—Measurements were made of the diameter of the cylinder and the bore of the cylinder of cores shown in thin sections and in freeze-etched preparations of *S. faecalis* strain XL and *S. faecalis* ATCC 8043. The dimensions were similar for both strains (Table 3). The diameters of the cores in freeze-etched cells are somewhat larger since the cells have not been fixed and dehydrated as they are in the preparation of thin sections.

Formation of cores during balanced growth—Culturing cells of *S. faecalis* strain XL under conditions in which the lag phase was eliminated and the optical density (O.D.) and dry weight of the cells were increasing

Fig. 13. Isolated core-like structures obtained from the disintegration of cells of *S. faecalis* strain XL in the Braun homogenizer after fixation for 30 min with 4% glutaraldehyde in the cold. (×30,000).

Fig. 14. Isolated core-like structures obtained from the disintegration of cells of *S. faecalis* strain XL in the Braun homogenizer after fixation for 30 min with 4% glutaraldehyde in the cold. (×23,300).

Fig. 15. Isolated core-like structures obtained from the disintegration of cells of *S. faecalis* strain XL in the Braun homogenizer while suspended in 0.1 M HCl-KCl buffer, pH 2.0. (×23,300)



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(15)

Table 3. *Diameters of Cores from Streptococcus faecalis ATCC 8043 and Streptococcus faecalis Strain XL*[a]

Bacterium	Glutaraldehyde[b] fixation		Glutaraldehyde-osmium[c] fixation		Unfixed[d] freeze-etched	
	Cylinder	Bore	Cylinder	Bore	Cylinder	Bore
S. faecalis ATCC 8043	0.129	0.041	0.148	0.054	0.153	0.078
S. faecalis strain XL	0.109	0.025	0.129	0.047	0.132	0.067

[a]Averages of measurements of 40 round cross sections.

[b]Fixation for 30 min to 2 hr with 2% glutaraldehyde in the cold.

[c]Fixation for 30 min with 2% glutaraldehyde in the cold and postfixation with 1% osmium tetroxide for 1 to 16 hr at room temperature.

[d]Unfixed cells suspended in 30% glycerol.

at the same rate resulted in the formation of cores in the cells at a predictable time in the growth cycle. Plots of the growth of *S. faecalis* strain XL (Fig. 16) demonstrate that growth of the culture begins without a lag phase. The formation of cores occurred after 3 hr of growth at an $O.D._{660}$ of 1.162 (Fig. 16). For the first hour of growth few mesosomes were observed (Fig. 17), but beginning at 2 hr of growth ($O.D._{660}$ of 1.094) extensive mesosomal membrane was observed in the cells (Fig. 18). The mesosomes became smaller after the formation of cores which occurred after 3 hr growth (Fig. 19). The observations of the formation of cores in cells from a culture of *S. faecalis* strain XL are summarized in Table 4.

Persistence of core structures and their relationship to viability of the cells—In cultures permitted to grow for 7 days in Todd-Hewitt broth at 37 C, cores and mesosomes could be observed throughout this period of growth (Figs. 20, 21, 22, and 23). In order to determine whether the formation of cores caused a decrease of viability in cells in the culture, plate counts were made of colony-forming-units (CFU) of *S. faecalis* strain XL (Table 5). Cultures of *S. faecalis* strain XL form short chains during logarithmic growth and occur mainly as pairs of dividing cells after this period. This chaining makes viable counts quite

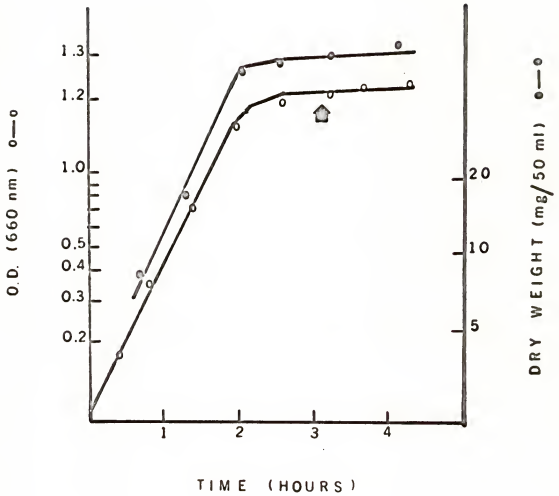


Figure 16. Growth of *Streptococcus faecalis* strain XL in Todd-Hewitt broth at 37 C, showing changes in (i) dry weight and (ii) optical density. Cores were first observed in the cells after 3 hr of growth (arrow) at an O.D. (660 nm) of 1.164 (Table 4).

Table 4. *Observation of the Formation of Mesosomes and Cores in Cells of Streptococcus faecalis Strain XL During Growth in Todd-Hewitt Broth at 37 C*

Time of Incubation (hr)	$O.D._{660\mu m}$	Dry weight (mg/50 ml)	Observations
0	0.830	-	-
0.25	0.122	-	-
0.5	0.166	3.54	No cores, septal mesosomes, large areas of nuclear material
0.75	0.235	-	-
1.0	0.334	7.50	-
1.25	0.519	-	No cores, septal mesosomes
1.5	0.675	15.50	No cores, many small mesosomes
2.2	1.094	24.54	No cores, extensive formation of mesosomes
2.3	1.107	-	-
2.5	1.125	25.18	No cores, many large mesosomes
3.0	1.164	-	Cores, smaller mesosomes
3.25	1.170	25.65	-
3.5	1.171	-	-
3.75	1.168	-	-
4.0	1.174	25.84	Cores, smaller mesosomes
4.5	1.180	-	-
8.0	1.185	25.54	Cores, many small mesosomes

Fig. 17. Thin section of cells of *S. faecalis* strain XL after growth for 1 hr in Todd-Hewitt medium at 37 C. Few mesosomes and no cores are observed at this time. (×43,700).

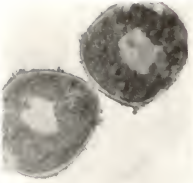
Fig. 18. Thin section of cells of *S. faecalis* strain XL after growth for 2½ hr in Todd-Hewitt broth at 37 C. Many mesosomes (arrows) are observed at this time. (×39,400).

Fig. 19. Thin section of a cell of *S. faecalis* strain XL after growth for 3 hr in Todd-Hewitt broth at 37 C. This is the initial time at which cores can be observed. (×57,600).

Fig. 20. Thin section of a cell of *S. faecalis* strain XL after growth for 4 hr in Todd-Hewitt broth at 37 C. The cells were fixed in sequence by: (i) prefixation in 0.01% OsO_4 for 5 min; (ii) fixation by 2% glutaraldehyde for 2 hr in the cold; and (iii) postfixation by 1% OsO_4 for 1 hr at room temperature. This fixation preserved both the cores and mesosomes (M) that are present in the cells. (×54,200).

Fig. 21. Thin section of cells of *S. faecalis* strain XL after growth for 13½ hr in Todd-Hewitt broth at 37 C. These cells are fixed by the Ryter-Kellenberger technique (102) which fixes the mesosomes well but not the cores. (×42,600).

Fig. 22. Thin section of *S. faecalis* strain XL after growth for 20 hr in Todd-Hewitt broth at 37 C. The cells were fixed for 30 min with 2% glutaraldehyde in the cold. (×24,800).



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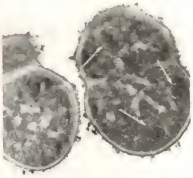

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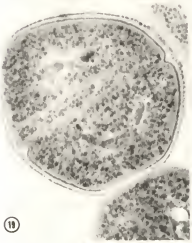

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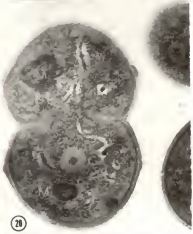

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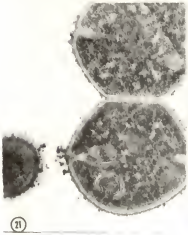

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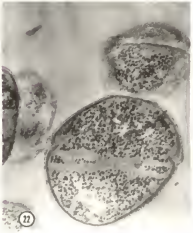

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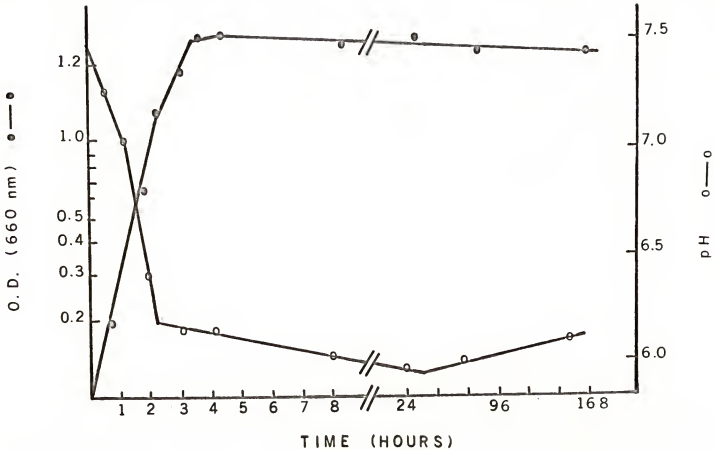


Figure 23. Growth of a culture of *Streptococcus faecalis* strain XL in Todd-Hewitt broth at 37 C for 7 days (168 hr) for the observation of the persistence of cores and mesosomes in cells in old cultures. Changes in optical density and pH are shown. Both cores and mesosomes could be observed in thin sections of cells throughout the 7 days of growth starting after 3 hr.

Table 5. *Determination of Colony-Forming-Units (CFU) in Relation to the Formation of Cores in Streptococcus faecalis Strain XL Growing in Todd-Hewitt Broth at 37 C*

Time of Incubation (hr)	$O.D._{660\ \mu m}$	pH of medium	Colony-Forming-units (CFU)/ml
0	0.104	7.4	-
0.25	0.138	-	-
0.5	0.192	-	8.2×10^7
1.0	0.371	7.0	2.9×10^8
1.5	0.734	-	1.0×10^9
1.75	0.948	-	1.0×10^9
2.0	1.095	6.2	-
2.5	1.187	-	9.2×10^8
3.0[a]	1.214	6.2	-
3.5	1.224	-	-
4.0	1.147	-	1.5×10^9
8.5	1.228	-	1.4×10^9
13.5	1.173	-	1.2×10^9
19.0	1.153	5.6	9.4×10^8
25.0	1.147	5.95	1.7×10^9
34.5	1.135	5.9	1.0×10^9
48.0	1.103	5.9	1.0×10^9
72.0	1.218	6.0	-
168.0	1.067	6.2	2.8×10^8

[a]Cores could be observed in cells beginning at this time.

inaccurate, but it can be ascertained from the data in Table 5 that no sharp decrease in colony-forming-units occurs, although approximately 90% of the cells contain cores after 3 hr of growth.

Effect of inhibition of protein synthesis on the formation of cores—Chloramphenicol (135) was added to growing cultures in a concentration of 50 μg/ml (89, 138). The chloramphenicol (CAP) was added 1½ hr and 30 min before the time that cores would be formed normally in the culture (Fig. 24). Control cells (Fig. 25) taken from a culture after 2½ hr incubation did not contain cores. At this point, 50 μg of CAP per ml was added to the culture and after 1½ hr incubation (Figs. 26 and 27) no cores were observed in the cells, although cells from a control culture grown the same length of time without CAP (Fig. 28) contained cores in a high percentage of the cells. There were some masses of clear material accumulated in the cells (Fig. 26) and some evidence of aberrant septation (Fig. 27). The cells treated with CAP (Figs. 26 and 27) were inoculated into fresh medium and into filtered, spent broth from a stationary culture and incubated for 24 hr. Cores were subsequently produced in these cells (Figs. 29 and 30) after removal from the presence of CAP. When 50 μg of CAP per ml was added to a growing culture 30 min before normal formation of cores, no cores were

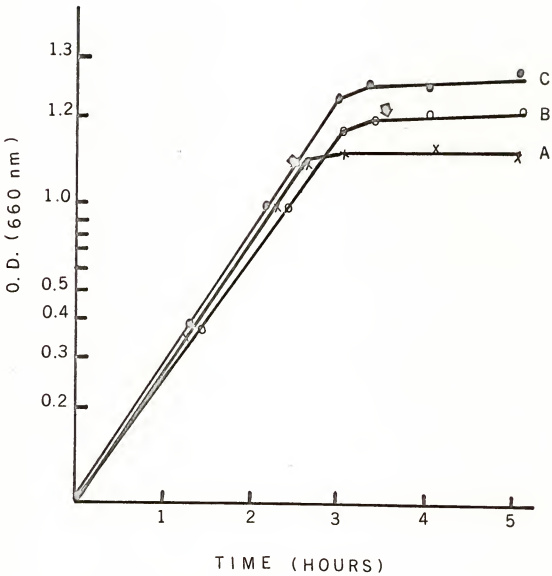
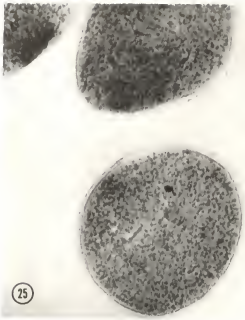


Figure 24. Growth of cultures of *Streptococcus faecalis* strain XL in Todd-Hewitt broth at 37 C after addition of 50 µg of chloramphenicol: (A) 1½ hr before the normal time for formation of cores, and (B) 30 min before this time. The control culture (C) was incubated in Todd-Hewitt broth without chloramphenicol.

Fig. 25. Thin section of control cells of *S. faecalis* strain XL grown to mid-log phase in Todd-Hewitt broth at 37 C and fixed for 30 min with 2% glutaraldehyde in the cold. No cores can be observed in the cells. (×67,700).

Fig. 26. Thin section of cells of *S. faecalis* strain XL from a culture to which 50 μg of chloramphenical (CAP) per ml was added for 1½ hr. No cores can be observed in the cells. (×61,000).

Fig. 27. Thin section of a cell of *S. faecalis* strain XL from a culture to which 50 μg of CAP per ml was added for 1½ hr after growth for 2½ hr. There is evidence of aberrant septal formation. No cores can be observed. (×47,700).



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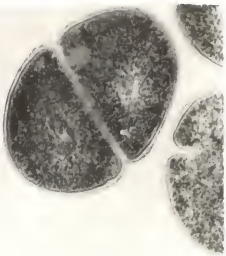

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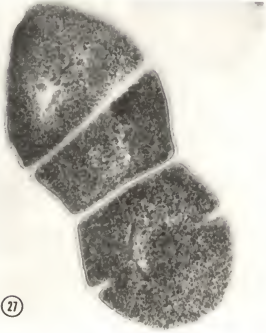

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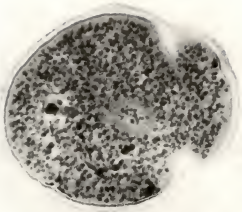
Fig. 28. Thin section of a control cell of *S. faecalis* strain XL from a culture in Todd-Hewitt broth incubated for 4 hr at 37 C and fixed for 30 min with 2% glutaraldehyde in the cold. There was extensive formation of cores in these cells. (×75,000)

Fig. 29. Thin section of a cell of *S. faecalis* strain XL exposed to 50 μg of CAP per ml for 1½ hr after growth for 2½ hr in Todd-Hewitt broth at 37 C and subsequent culturing for 24 hr at 37 C in fresh Todd-Hewitt broth. The ability to form cores was not lost by growth in the presence of CAP. (×75,000)

Fig. 30. Same as Fig. 29 except that the cells were cultured subsequently for 24 hr in spent Todd-Hewitt broth, pH 5.9, which was filtered from a stationary culture. Cores were formed. (×92,700)

Fig. 31. Thin section of cells of *S. faecalis* strain XL from a culture growing in Todd-Hewitt broth to which 50 μg of CAP per ml was added 30 min prior to the time or normal formation of cores. No cores were formed. (×58,800)



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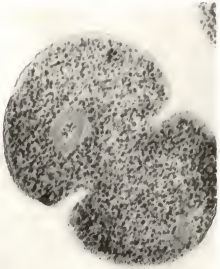

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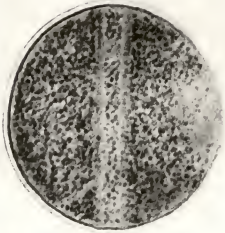

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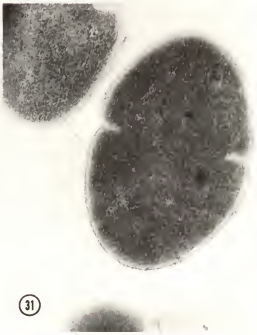

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formed (Fig. 31), although numerous cores were observed in cells from control cultures incubated for 5 hr without CAP (Fig. 32).

These results indicate that cores are not self-assembled from preexisting pools of protein in the cells but rather are formed from newly synthesized protein or by an enzymic process whose formation is inhibited by CAP.

Conditions of formation and disaggregation of cores—Cells from cultures that are growing logarithmically do not contain cores. This suggests that cores disappear from the cells at some time after inoculation into fresh broth. Cores initially observed in cells (Fig. 33) can no longer be seen 5 min after the cells are inoculated into fresh medium (Fig. 34). These cells were centrifuged at 37 C in order to avoid a temperature shift in the culture that might influence the time of disintegration of the cores.

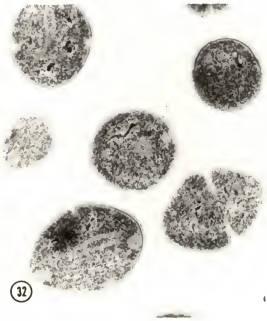
Cores are stable in cells incubated for 60 min at 2 C (Fig. 35). Cores also remain stable in the cells after introduction into a filtered, spent medium at pH 6.1 (Table 6). If young cells, which do not contain cores, are introduced into spent broth, no cores form within a 3 hr period even though the cells grow slowly. These findings are summarized in Table 6 and indicate that

Fig. 32. Thin section of control cells of *S. faecalis* strain XL cultured for 5 hr in Todd-Hewitt broth at 37 C. Normal formation of cores occurred. (×25,000)

Fig. 33. Thin section of a cell of *S. faecalis* strain XL before introduction into fresh Todd-Hewitt broth. (×60,800)

Fig. 34. Thin section of cells of *S. faecalis* strain XL 5 min after introduction into fresh Todd-Hewitt broth, pH 7.2 at 37 C. The cores can no longer be observed in the cells. (×35,400)

Fig. 35. Thin section of a cell of *S. faecalis* strain XL cultured for 16 hr in Todd-Hewitt broth + 1% glucose at 37 C and then incubated for 60 min at 2 C. The cores remain stable in the cold. (×56,200)



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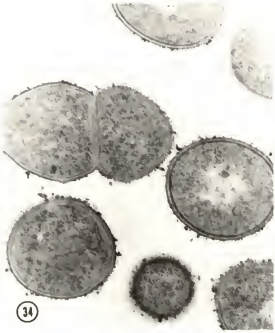

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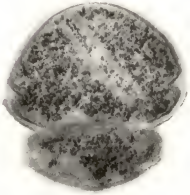

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Table 6. *Changes in Cells of Streptococcus faecalis Strain XL With and Without Cores After Inoculation Into Fresh and Spent Todd-Hewitt Broth at 37 C*

Cells	Broth	Cores
With cores	Fresh medium, pH 7.2	Disappear within 5 min
With cores	Fresh medium, pH 6.1	Remain stable in the cells
With cores	Spent broth, pH 6.1	Remain stable in the cells
Without cores	Spent broth, pH 6.1	No cores formed within 3 hr
Without cores	Fresh medium, pH 6.1	No cores formed within 3 hr

neither pH alone nor conditions nor factors existing in spent broth induce the formation of cores, although pH is apparently an important parameter in the production of cores.

Growth in buffered media—In control cultures, the pH dropped to 6.2 after 2 hr of growth and was still 6.2 at 3 hr when cores were formed initially (Figs. 36 and 37). A culture of *S. faecalis* ATCC 8043 was buffered in Todd-Hewitt broth with 0.02 M potassium phosphate buffer, pH 7.4 and incubated at 37 C. The growth was slower than normal in this buffered medium, but after 48 hr of growth, there was still no production of cores at a final pH of 6.6 (Fig. 38). When cells in the buffered medium were incubated for an additional 1½ hr at 37 C after the pH of the medium had been lowered to 4.4, no formation of cores occurred (Fig. 39), although cores formed readily in control cultures that were not buffered (Fig. 37).

Two other cultures buffered with 0.01 M citrate buffer, pH 4.51 and 0.01 M Tris buffer, pH 7.9 did not produce cells that formed cores in either medium (Figs. 40 and 41). The pH did not become low enough (pH 6.52) for formation of cores in the culture buffered with Tris buffer, and the growth was greatly diminished in the culture buffered with citrate buffer (Fig. 36).

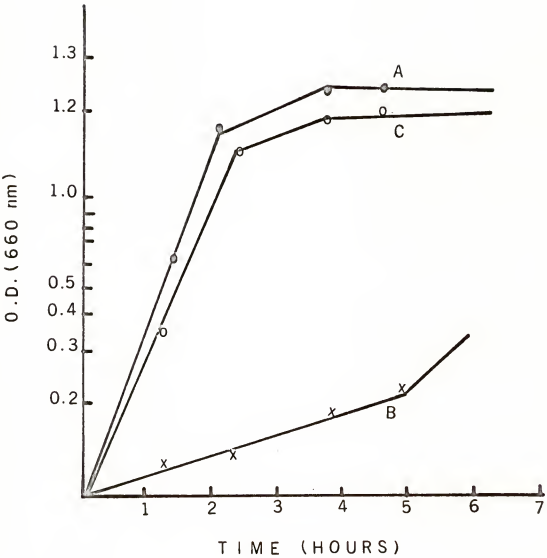


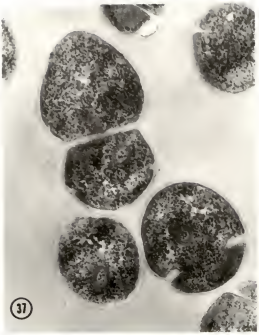
Figure 36. Growth of cultures of *Streptococcus faecalis* strain XL in (A) Todd-Hewitt broth, unbuffered; (B) Todd-Hewitt broth buffered with 0.01 M citrate buffer, pH 4.5; and (C) Todd-Hewitt broth buffered with 0.01 M Tris buffer, pH 7.9. Cultures were incubated at 37 C.

Fig. 37. Thin section of control cells of *S. faecalis* strain XL cultured for 14 hr in unbuffered Todd-Hewitt broth at 37 C to a final pH of 5.6. Normal formation of cores occurred. (×32,800)

Fig. 38. Thin section of cells of *S. faecalis* ATCC 8043 cultured for 16 hr at 37 C in Todd-Hewitt broth buffered with 0.02 M potassium phosphate buffer, pH 7.4. The final pH was 6.6. No cores were formed. (×36,800)

Fig. 39. Thin section of cells of *S. faecalis* ATCC 8043 which were cultured for 16 hr at 37 C in Todd-Hewitt broth buffered with 0.02 M potassium phosphate buffer, pH 7.4 and then incubated for an additional 1½ hr at 37 C after the pH of the medium was lowered to 4.4. No cores can be observed to form. (×45,000)

Fig. 40. Thin section of cells of *S. faecalis* strain XL cultured for 4½ hr at 37 C in Todd-Hewitt broth buffered with 0.01 M Tris buffer, pH 7.9. Final pH was 6.6. No cores were formed. (×34,400)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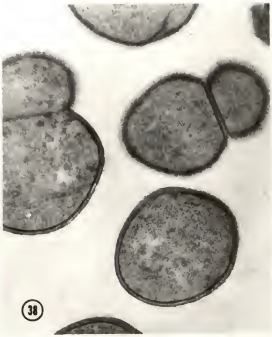
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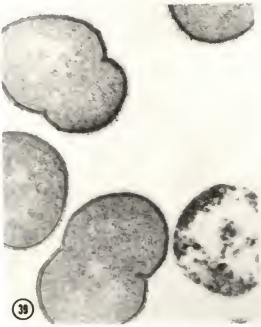
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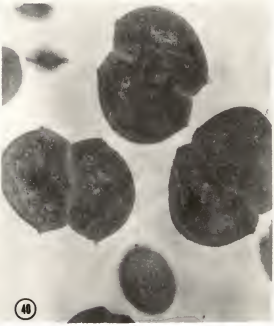
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Effect of pH on the stability of cores—It was observed that cores were not stable at pH's above 6.5 and that they disintegrated rapidly when the pH was raised above 7.0. Cells of *S. faecalis* ATCC 8043 in 0.1 M HCl-KCl buffer, pH 2.0 incubated for 60 min at 37 C (Fig. 42) are stable at this pH even though much of the cytoplasm is lost. A cell incubated for 30 min at 37 C in 0.1 M citrate buffer, pH 4.0 (Fig. 43) also contained stable cores. In distilled water at pH 6.0 (Fig. 44) the cores are clearly discernible after incubation for 30 min at room temperature. When the pH of the culture medium was raised from pH 6.1 to pH 7.0 after induction of cores had occurred and the cells incubated for 30 min at 37 C, the cores were no longer visible (Fig. 45). Cells containing cores that were incubated for 30 min at 37 C in 0.1 M Tris buffer, pH 7.2 (Fig. 46) no longer contained cores but myelin-like mesosomes were present. The cores were disaggregated after 30 min at 37 C in 0.1 M potassium phosphate buffer, pH 8.0 (Fig. 47) and the same phenomenon was observed after raising the pH of the medium to pH 8.0 (Fig. 48) and incubating the culture for 30 min at 37 C. When the pH of the medium was raised to pH 8.0 and the culture chilled to 2 C for 30 min rather than incubated at 37 C, the core structures disappeared (Fig. 49) indicating that they are disrupted at pH 8.0 whether the cells are at growth temperature or in the cold. Even

Fig. 41. Thin section of a cell of *S. faecalis* strain XL cultured for 4½ hr at 37 C in Todd-Hewitt broth buffered with 0.01 M citrate buffer, pH 4.5 for a final pH of 4.73. No cores were observed. (×37,100)

Fig. 42. Thin section of cells of *S. faecalis* ATCC 8043 incubated for 60 min in 0.1 M HCl-KCl buffer, pH 2.0. The cores (arrows) were stable at this low pH. (×60,000)

Fig. 43. Thin section of a cell of *S. faecalis* ATCC 8043 incubated for 60 min in 0.1 M citrate buffer, pH 4.0. The cores (arrows) remained stable. (×57,300)

Fig. 44. Thin section of cells of *S. faecalis* ATCC 8043 incubated for 30 min in distilled water at pH 6.0. The cores are clearly observed in the cells. (×45,400)

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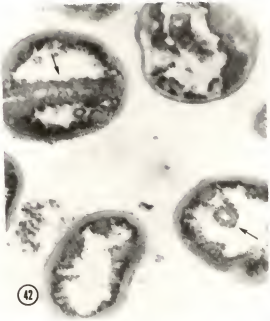

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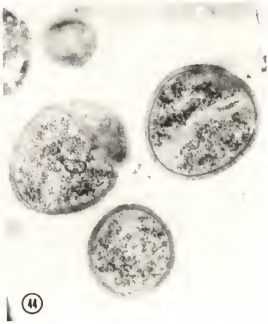

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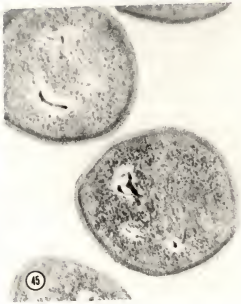
Fig. 45. Thin section of cells of *S. faecalis* ATCC 8043 in Todd-Hewitt broth after the pH was raised from 6.1 to 7.2, following the induction of cores, and the culture incubated for an additional 30 min at 37 C. Cores were no longer visible in the cells after incubation at the higher pH. (×74,400)

Fig. 46. Thin section of a cell of *S. faecalis* ATCC 8043 taken from a culture in stationary growth after the formation of cores and incubated for 30 min at 37 C in 0.1 M Tris buffer, pH 7.2. Cores are no longer observed in the cells. (×91,000)

Fig. 47. Thin section of a cell of *S. faecalis* ATCC 8043 taken from a culture in stationary growth phase after the formation of cores and incubated for 30 min at 37 C in 0.1 M Tris buffer, pH 8.0. Cores are no longer observed. (×75,000)

Fig. 48. Thin section of cells of *S. faecalis* ATCC 8043 cultured to the stationary growth phase after which the pH of the medium was raised to 8.0, and the culture incubated for an additional 30 min at 37 C. Cores were no longer visible. (×72,500)



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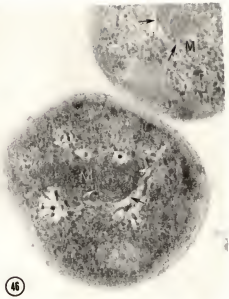

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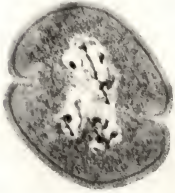

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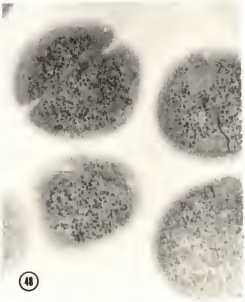

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after a brief washing of 5 min in 0.1 M Tris buffer, pH 8.2 in 0.5 M sucrose, the cores are barely discernible (Fig. 50). These observations indicate that core structures are not stable above pH 6.5 and remain stable at pH's as low as 2.0

Formation of cores in autolyzing cells—Young cells from logarithmically growing cultures are never observed to contain cores. However, when young cells are permitted to autolyze in 0.01 M potassium phosphate buffer, pH 6.5 at 37 C, cores are formed as autolysis proceeds.

In the initial stages of autolysis of cells of *S. faecalis* ATCC 8043 (Figs. 51 and 52) large accumulations of granular material form in the septal area where the mesosomes which were connected to the septa appear to have dislodged from their sites of attachment. Cores can be observed after 30 min autolysis at pH 6.5 in cells of *S. faecalis* strain XL (Figs. 53, 54, and 55). A layered structure can be observed in the newly formed cores (Fig. 55).

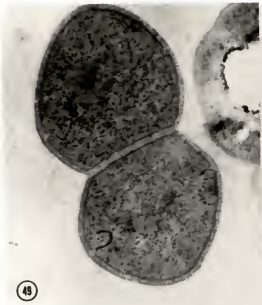
Formation of cores in the presence of penicillin—In addition to autolysis, the formation of cores in young cells can be induced by growing them in the presence of penicillin. The addition of 1000 units of penicillin G per ml to cultures of *S. faecalis* strain XL at the middle of the logarithmic growth phase will induce the formation of cores in the cells within 30 min (Fig. 56).

Fig. 49. Thin section of a cell of *S. faecalis* ATCC 8043 cultured to the stationary phase at pH 5.9 after which the pH of the medium was raised to 8.0 and the culture incubated for an additional 30 min at 2 C. Cores were no longer visible. (×72,000)

Fig. 50. Thin section of core-containing cell of *S. faecalis* ATCC 8043 washed for 5 min with 0.1 M Tris buffer, pH 8.2 in 0.5 M sucrose. The core is barely discernible. (×71,500)

Fig. 51. Thin section of a young cell of *S. faecalis* ATCC 8043 taken from a logarithmically growing culture and permitted to autolyze for 30 min in 0.01 M potassium phosphate buffer, pH 6.5, at 37 C. Areas of granular material (arrows) are seen near the septum. (×37,200)

Fig. 52. Thin section of a young cell of *S. faecalis* ATCC 8043 taken from a logarithmically growing culture and permitted to autolyze for 30 min in 0.01 M potassium phosphate buffer, pH 6.5, at 37 C. Areas of granular material (arrows) and a core can be observed in the cell. (×61,000)



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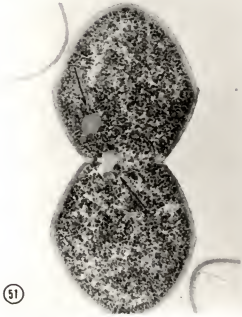

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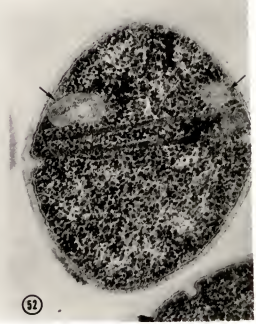

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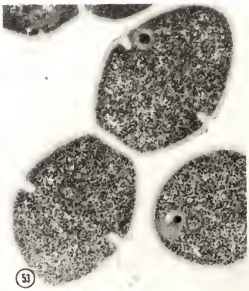
Fig. 53. Thin section of cells of *S. faecalis* strain XL from a logarithmically growing culture after autolysis for 30 min at 37 C in 0.01 M phosphate buffer, pH 6.5. (×57,000)

Fig. 54. Thin section of a cell of *S. faecalis* strain XL from a logarithmically growing culture after autolysis for 30 min at 37 C in 0.01 M phosphate buffer, pH 6.5. A core (arrow) can be seen forming in the cell. (×47,000)

Fig. 55. Thin section of a cell of *S. faecalis* strain XL from a logarithmically growing culture after autolysis for 30 min at 37 C in 0.01 M phosphate buffer, pH 6.5. A core (arrow) can be seen forming in the cell. (×60,000)

Fig. 56. Thin section of cells of *S. faecalis* strain XL from a logarithmically growing culture 30 min after the addition of 1000 units of penicillin G per ml. (×32,200)



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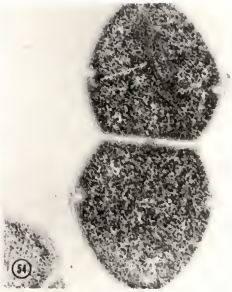

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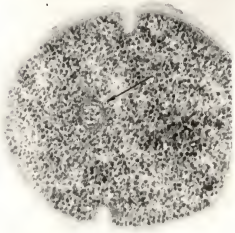

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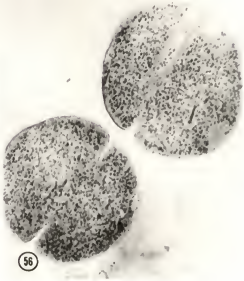

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Structure of cores in spheroplasts—When cells of *S. faecalis* strain XL were treated with 250 μg of lysozyme per ml at 37 C in distilled water at pH 6.0 to form spheroplasts, it was discovered that the cores within the spheroplasts began to disintegrate after even 5 min treatment with lysozyme. The disintegrating cores appeared to have layers of dense material (Figs. 57 and 58) which were observable in thin sections of cells of *S. faecalis* strain XL after treatment for 30 min with lysozyme. It is difficult to distinguish between the cross sections of cores that are shown and mesosomes, except that mesosomes are generally extruded during spheroplasting and are concise, tightly coiled structures (Fig. 59) when they can be observed in the spheroplasts. This apparent substructure in the disintegrating cores is similar to that observed in the newly formed cores in cells undergoing autolysis (Fig. 56). The layered material is quite dense and in some areas appears to be bilayered like membrane (Figs. 57 and 58). The layered structure is unmistakable in longitudinal section (Fig. 60).

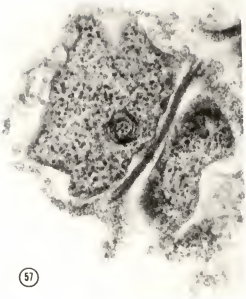
The substructure in the cores of spheroplasts was also quite evident in freeze-etched preparations of unfixed spheroplasts (Figs. 61, 62, and 63). A freeze-etched preparation of a mesosome in a young cell from a logarithmically growing culture (Fig. 64) is quite

Fig. 57. Thin section of a spheroplast of *S. faecalis* strain XL formed by exposure to 250 μg of lysozyme per ml in distilled water at pH 6.0 for 30 min at 37 C. The structure in the cell appears to be a disintegrating core. (×82,500)

Fig. 58. Thin section of a spheroplast of *S. faecalis* strain XL formed by exposure to 250 μg of lysozyme per ml in distilled water for 30 min at 37 C. The structure in the cell appears to be a disintegrating core. (×77,200).

Fig. 59. Typical morphology of a mesosome in a thin section of a spheroplast of *S. faecalis* strain XL formed by exposure to 250 μg of lysozyme per ml for 30 min at 37 C in distilled water, pH 6.0. (×48,800)

Fig. 60. Longitudinal section of an apparently disintegrating core in a spheroplast of *S. faecalis* strain XL formed by exposure to 250 μg of lysozyme per ml for 30 min at 37 C in distilled water, pH 6.0. (×63,400)



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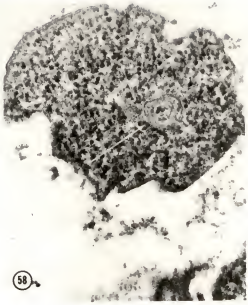

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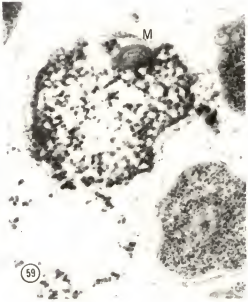

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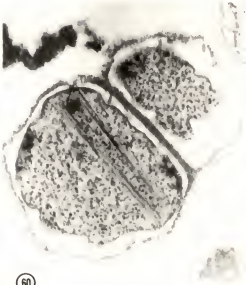

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Fig. 61. Freeze-etched, unfixed spheroplast of *S. faecalis* strain XL showing the structure of the core. (×66,000)

Fig. 62. Freeze-etched, unfixed spheroplast of *S. faecalis* strain XL showing the structure of the core. (×64,400)

Fig. 63. Freeze-etched, unfixed spheroplast of *S. faecalis* strain XL showing the structure of the core. (×83,000)

Fig. 64. Freeze-etched, unfixed, young cell of *S. faecalis* strain XL showing the typical morphology of a mesosome in contrast to the cross section of a core. (×100,000)

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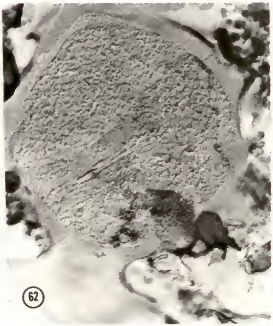
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similar in appearance to the disintegrating core (Fig. 63). Often forms are seen (Fig. 65) that could represent intermediates in the formation of cores.

Cells treated with lysozyme in distilled water and placed in the mixture used by Dirksen (26) at pH 6.0 for stabilizing microtubules are not observed to contain cores (Fig. 66) indicating that cores are not stabilized under the same conditions as are microtubules. When spheroplasts were formed by the action of lysozyme on cells suspended in 0.01 M Tris buffer, pH 8.4 as shown in a cell of *S faecalis* ATCC 8043 (Fig. 67), no cores were observed in the cells after 5 min. The cores apparently disintegrated rapidly in spheroplasts at this higher pH.

Occasionally crystalline inclusions were seen in thin sections of core containing cells of *S. faecalis* ATCC 8043 (Fig. 68). These have not been reported before in group D streptococci and their relationship to core formation or other cellular processes are unknown. They are similar to the proteinaceous inclusions in *Bacillus thuringiensis* (86) and the viral inclusions in *Escherichia coli*, (7) although the latter have a wider crystalline lattice.

Cytochemical studies of core structures— Cytochemical techniques were used in order to determine the general chemical nature of cores. The silver

Fig. 65. Freeze-etched, unfixed cell of *S. faecalis* strain XL showing a formation that may represent an intermediate form in the formation of cores. (×67,000)

Fig. 66. Thin section of cells of *S. faecalis* strain XL after treatment for 30 min with 250 μg of lysozyme per ml at 37 C in distilled water at pH 6.0 followed by lysis in the mixture Dirksen (26) used for stabilizing microtubules: 0.1 M EDTA, 0.5 M sucrose, and 0.15 M dithiodipropanol. No cores were observed to remain after this treatment. (×47,700)

Fig. 67. Thin section of spheroplasts of *S. faecalis* strain XL after treatment with 250 μg of lysozyme per ml for 30 min at 37 C in 0.01 M Tris buffer, pH 8.4. No cores were visible in spheroplasts formed at this high pH. (×47,200)

Fig. 68. Thin section of cells of *S. faecalis* ATCC 8043 in which unusual crystalline-like inclusions can be seen. (×62,000. Insert, ×94,200)

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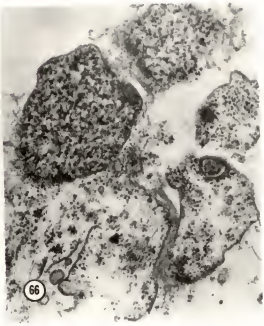

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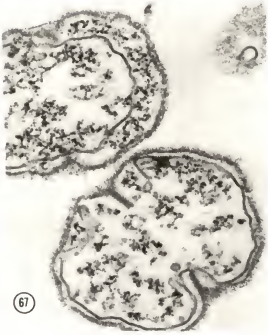

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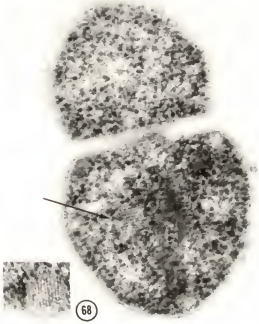

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methenamine staining procedure was used for the detection of polysaccharides containing 1,2-glycol groups and the results (Figs. 69, 70, 71, and 72) indicate that cores do not contain polysaccharides of these specifications. The heavy staining of the polysaccharides in the cell wall of *S. faecalis* strain XL can be observed in these stained preparations. An analysis of the chemical composition of isolated cell walls of *S. faecalis* strain XL (Table 7) shows that rhamnose and glucose make up a large part of the wall. These sugars would be stainable by the silver methenamine method as would galactose and ribitol. The analysis of the cell wall of *S. faecalis* strain XL is similar to the data reported by Bleiweis et al. (4) on a related type I group D streptococcus D76. The results indicate that the cores do not contain polysaccharide stainable by the silver methenamine method and, therefore, are unlikely to contain components of the cell wall.

Digestion of cores from thin sections by enzymes—Of the enzymic digestions attempted on thin sections of *S. faecalis* strain XL, those involving deoxyribonuclease, lipase, and trypsin did not produce definitive results. Ribonuclease seemed to digest the ribosomes without affecting the cores, but the results were variable.

In contrast to the control cells (Fig. 73) in which the cores were still visible, digestion of the

Fig. 69. Thin section of *S. faecalis* strain XL stained for 20 min by the silver methenamine staining procedure. The core does not stain, and the cell wall stains heavily. (×47,200)

Fig. 70. Longitudinal section of *S. faecalis* strain XL stained for 20 min by the silver methenamine staining procedure. (×72,700)

Fig. 71. Cross section of a core in a thin section of *S. faecalis* strain XL stained for 20 min by the silver methenamine staining procedure. (×63,000)

Fig. 72. Thin section of cells of *S. faecalis* strain XL stained for 20 min by the silver methenamine staining procedure. (×34,000)

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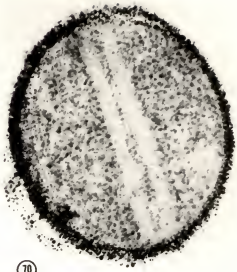

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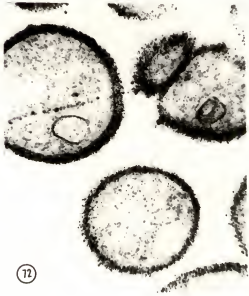

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Table 7. *Composition of Cell Walls of Streptococcus faecalis Strain XL*

Component	μ moles/mg	% Composition[a]
Rhamnose	1.20	19.7
Glucose	1.06	19.1
Galactose	0.02	0.4
Glucosamine	0.30	6.6
Galactosamine	0.34	7.5
Muramic acid	0.29	8.5
Alanine	0.76	6.8
Lysine	0.37	5.4
Glutamic acid	0.34	5.0
Aspartic acid	0.28	3.7
Phosphorus	0.44	2.8
Ribitol	0.44	6.7

[a]Represents 92.2% of the total dry weight of the cell wall.

cores by pronase could be seen clearly in thin sections of cells embedded in glycol-methacrylate. In a thin section of *S. faecalis* strain XL that was digested for 45 min by pronase (Fig. 74), the cores became transparent as they were removed from the section by the action of the enzyme. The glutaraldehyde-fixed cores were not removed from control thin sections treated with buffers alone.

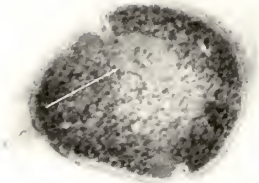
Control cells (Fig. 75) retain the cell wall, but digestion of sections of *S. faecalis* strain XL embedded in glycol-methacrylate by 2500 μg of lysozyme per ml (Fig. 76) indicates that the cell wall is digested and removed by the enzyme, but the cores are not. This result indicates, as already suggested by the absence of polysaccharide in the structures, that the cores are not composed of cell wall material.

Fig. 73. Thin section of a control cell of *S. faecalis* strain XL embedded in glycol-methacrylate and oxidized for 5 min with 3% hydrogen peroxide. (×85,400)

Fig. 74. Thin section of a cell of *S. faecalis* strain XL after digestion for 45 min with 0.2% pronase. The cores are digested from the sections by the enzyme. (×27,200)

Fig. 75. Thin section of a control cell of *S. faecalis* strain XL embedded in glycol-methacrylate and oxidized for 5 min with 3% hydrogen peroxide. (×56,500)

Fig. 76. Thin section of a cell of *S. faecalis* strain XL embedded in glycol-methacrylate and digested for 2 hr with 2500 μg of lysozyme per ml followed by treatment with 0.2% sodium lauryl sulfate for 10 min. The wall is digested but the core is not. (×63,000)



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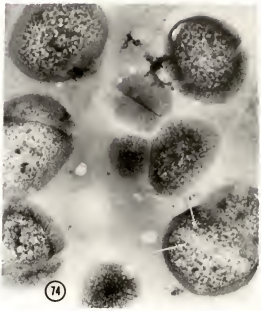

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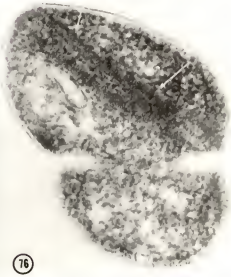

76

DISCUSSION

A clear definition of the structure of cores by freeze-etching was a necessary first step in studying the ultrastructure of the core as well as the factors that govern the formation and disintegration of the structure. The process of chemical fixation often induces artifactual appearances in structures that are difficult to clarify unless unfixed cells can be observed by some technique such as freeze-etching. The fact that cores existed in the same form in unfixed cells as they did in chemically fixed material established a basis for further work on their stability and other properties.

It is curious that such a regular, cylindrical structure as the core does not have discernible subunits. Most of the other tubular structures in procaryotic cells such as gas vacuoles (6, 13), spore appendages (91, 139, 140), and rhapidosomes (71, 72, 137) have a regular arrangement of subunits that may vary somewhat in pitch or conformation from species to species. Except for the even, cylindrical shape, cores seem to be a homogeneous structure, which may be responsible for the difficulties encountered in attempting to isolate them. In the absence of subunits, however, it is interesting that layers of

substructural material could be observed both in cores forming in autolyzing cells and in cores disintegrating in spheroplasts. Other cylindrical structures in procaryotes, as mentioned above, are formed from subunits and not from a coalescing of thin sheets of material.

In the original observations of McCandless et al. (74) on core-like structures in group D streptococci, the authors described a nontubular type of core in parent cells of the streptococci (see Figs. 1, 2, 3, and 4) and a tubular form with an electron-dense shell in stable protoplasts. No evidence has been found in this study for more than one type of core. The "tubular" core probably corresponds to the disintegrating core-like forms that are reported here in spheroplasts of *Streptococcus faecalis* strain XL formed by treatment with lysozyme. McCandless et al. (74) also suggested that the tubular core may be derived from the nontubular type during cellular aging. They postulated that the electron-dense shell of the tubular form may result from condensation due to polymerization or cross-linking (or both) in the nontubular core. In this study, however, rather than a single electron-dense shell, several layers of such material were observed both in newly forming cores in cells that were autolysing and also in spheroplasts in which the cores appeared to be disintegrating. Thus, it would seem that the layered material represents an intermediate in the formation of cores.

McCandless et al. (74) also reported that occasionally the ribosomes within the bore of the core appeared to have a helical configuration. Although this was not observed generally in this study, it raises the consideration of whether cores might be formed into a cylindrical conformation because the components of the core are formed around the ribosomes or whether the ribosomes may be involved in the synthesis of the core. For example, the chain or group of ribosomes down the center of the core may be a system of some specialized synthetic capacity that is still producing a product (i.e., structural or enzymic protein) that forms into a core after active cell division has stopped.

Another observation that might be applicable to this hypothesis is that made by Higgins and Daneo-Moore (51) who reported that the synthesis of mesosomal membrane in *S. faecalis* ATCC 9790 depends upon the rate of synthesis of deoxyribonucleic acid (DNA). During active, logarithmic growth, large amounts of mesosomal membrane are produced, just as observed here in *S. faecalis* strain XL. When the cultures reach the stationary growth phase, the rate of DNA synthesis slows. It was shortly after this period that cores were first observed in cells of *S. faecalis* strain XL and that smaller mesosomal structures were seen.

This raises the question of whether group D streptococci may carry a genetic defect in the regulation

of the synthesis of some cellular constituent. All of the conditions (stationary growth, autolysis, and growth in penicillin) that cause the formation of cores also lead to inhibition of some synthetic activities. In addition, autolysis (52) and growth in penicillin (34) both cause initial damage in the septal area that causes the mesosomes attached to this site to be dislodged. In *S. faecalis* ATCC 8043, areas of material that appear to be mesosomal in origin appear after 30 min of autolysis in phosphate buffer (Figs. 51 and 52). After 30 min the formation of cores can be observed (Fig. 53). It can be speculated further that mesosomal membrane in group D streptococci has a structural specificity that causes it to depolymerize and reform in a tubular configuration. The mesosomes in *S. faecalis* (51) occur in lamellar layers in a bag-like shape that could be visualized as elongating into a tube. The problem involved in recognizing an intermediate stage with certainty makes this difficult to prove. The structure shown in the freeze-etched preparation in Fig. 65 is observed often and could represent such an intermediate.

The work reported by Higgins and Shockman (53) supports the possibility of a genetic defect in the synthesis of membrane by group D streptococci. They observed that after starvation for amino acids in *S. faecalis*

ATCC 9790 there was a rapid increase during the first 1 to 2 hr of starvation in the thickness of the cell wall and in the size of the nucleoid and the mesosomal membranes. When the cells were starved for threonine, mesosomal membrane accumulated for the first hour and then was degraded.

Perhaps the most important finding of this study has been that of the inhibition of the formation of cores by the cessation of protein synthesis in the presence of chloramphenicol (CAP). The production of cores does not occur when synthesis of protein is inhibited at 30 min before the time of formation of cores or during the logarithmic growth phase at 1½ hr before the normal time for formation of cores. It is apparent that cores are formed either: (i) from protein that is newly synthesized shortly before the cores are formed, or (ii) by an enzymic process that is inhibited when the synthesis or activation of the enzyme(s) involved is prevented by the presence of CAP. If cores were produced by a simple self-assembly from pools of protein precursors already present in the cells, they probably would be formed even in the presence of CAP.

Almost no mesosomes were observed in the cells after incubation with CAP. In *Escherichia coli* (W. E. C. Bradley and L. Astrachan, Absts. of the Annual Meeting of the American Society for Microbiology, p. 206, 1972), it

was reported that the synthesis of phospholipid was inhibited within 3 to 4 min after addition of 20 μg of CAP per ml to a logarithmically growing culture.

It would be incorrect to assume that cores are formed from a protein that is produced only in the early stationary phase of growth. Cells from young, logarithmically growing cultures will form cores if they are permitted to autolyze, or when penicillin is added to the culture, so that apparently the specific protein that goes into the core is present in the cells during logarithmic growth but is arranged into a core only when growth is interrupted. This does not happen when protein synthesis is inhibited, indicating that some factor involved in the formation of cores must be affected by CAP. There are several possible explanations for this. First, perhaps there is either an enzyme(s) or structural protein involved that must be produced *de novo* as the core is formed. Second, mesosomal membrane is rarely seen in cells treated with CAP, and cores would not be formed if they were derived from mesosomal membrane whose synthesis was inhibited by CAP. The third possibility is derived from the work of Pooley and Shockman (89) who found over an 80% decrease in autolytic activity of cells of *S. faecalis* ATCC 9790 within 10 min after treatment of cells with 100 μg of CAP per ml. If cellular autolysis is

involved in the production of cores, then CAP could be inhibiting the process by interfering with autolysis.

Experimental approaches that would be possible for clarifying the derivation of the cores are: (i) to incubate logarithmically growing cells in the presence of CAP, wash the antibiotic from the cells, and permit the cells to autolyze in order to observe whether they can form cores after CAP is removed, and (ii) to pulse-label cells with a radioactive amino acid during logarithmic growth, and just before time for cores to form. The presence of label would be determined first in the mesosomes, and subsequently in the cores after they were formed. This could be accomplished by isolating the structures or by use of autoradiographic techniques, observing the cores at pH 2.0 where most of the cytoplasm is removed from the cell, leaving the cores. The antibiotic actinomycin D would also be useful in these studies in that it completely inhibits the formation of ribonucleic acid (RNA) that is DNA-dependent.

Attempting to link the presence of glycerol teichoic acid to the formation of cores in group D streptococci is rather tenuous since many bacteria contain glycerol teichoic acids, including the homolactic group N streptococci which neither McCandless et al. (75) nor this author found to contain cores. The suggestion has been

made by Heptinstall et al. (50) that since teichoic acids are sometimes absent from membranes prepared from protoplasts (47, 110, 133), they may be either loosely associated with the membrane or they may be associated with the mesosomal membrane. This would support the speculation that the specificity for the core-like structure could come from a teichoic acid-membrane complex.

Heptinstall et al. (50) speculated that the widespread occurrence of teichoic acids in bacteria is evidence for their importance in the maintenance of cellular activity. Gram-negative bacteria do not contain teichoic acid, but they do contain lipopolysaccharides which possess sugar residues, phosphate groups and basic amino acids which may serve a function similar to teichoic acids. It has been observed by means of freeze-etched preparations (32) that the pattern of proteinaceous particles on the cytoplasmic membrane of *Escherichia coli* changed markedly when the cells were starved for magnesium. This finding would complement the work of Heptinstall et al. (50) in which they discovered that decreasing the amount of teichoic acid in the cell wall of *Staphylococcus aureus* resulted in a lowered binding of magnesium ion. They proposed that the most important function of teichoic acid in walls and membranes was the maintenance of a high concentration of bivalent cations in the region of the membrane.

The successful isolation and chemical characterization of these labile cores will perhaps answer the question of whether they are derived from mesosomal membrane and whether they contain teichoic acid within the structure. This will necessitate concurrent isolation and characterization of mesosomal membranes. It has taken considerable effort to isolate the few cores that have been observed structurally by negative staining for this study. The isolation of the quantities necessary for chemical analysis and for studies of the properties of the protein will be a difficult task.

Other workers have observed tubular structures in streptococci and other bacteria to which they attributed a membranous origin. Kuhrt and Pate (M. F. Kuhrt and J. L. Pate, Bacteriol. Proc., p. 165, 1971) observed large amounts of mesosomal membrane in cells of *Chondrococcus columnaris* which upon lysis released large numbers of small microtubular structures which they concluded, from a comparison of the chemical compositions, were derived from the mesosomal membrane. In group D streptococci, Corfield and Smith (20) postulated that the microtubular structures that they observed in L-forms of *S. faecalis* var. zymogenes could be aberrant forms of the normal mesosome which was no longer observed in bacterial protoplasts and L-forms. These observations

all raise the possibility that tubular structures may be derived from membrane and lend some support to the hypothesis derived from the results of this study.

The observation by Hubert et al. (61) of large core-like structures in L-forms of *Pseudomonas aeruginosa* is difficult to explain at this point until further work is performed on the phenomenon of the formation of cores. The authors admit that even though the L-form and parent form of *P. aeruginosa* were similar in properties, it was difficult to eliminate the possibility of contamination from the L-forms of group D streptococci that were also propagated in their laboratory.

It is possible that enzymic activity may be detected in cores, or that they may resemble microtubules in some respects (66, 104). Stephens (116) has reported the characterization of a 22S protein from the microtubules of the mitotic apparatus in sea urchin eggs. Under acid conditions, in the presence of magnesium ion, the 22S particles form filaments of 4 to 6 nm. These structures must be isolated at a slightly acid pH and dissolve rapidly in 0.6 M KCl. It will be necessary to isolate cores in a native state before such studies of the physical properties of the protein can be attempted. It was found in this study that cores are stable in the cold, unlike microtubules. However, they do resemble

microtubules in their rapid disaggregation and formation and in their stability at low pH.

Among the other possibilities that should be considered for the origin of the core is that of viral induction, although there is no evidence of virus-like structure within the core. Since cores occur at a specific period of the growth cycle, are found in almost every cell in the culture, and are found in all group D streptococci, it would be difficult to imagine that they are bacteriophage.

Another possibility for consideration is that the cores could be an accumulation of a protein similar to the toxic parasporal protein that accululates in *Bacillus thuringiensis* (86). This protein, however, is in the form of a crystalline inclusion. Although similar inclusions are seen in cells of *S. faecalis* ATCC 8043 (Fig. 68) there is no indication that they are related to the formation of cores, and they may represent accumulations of some substance induced by the process of autolysis.

There is a model proposed from the work of Ghosh et al. (39) which indicates that penicillinase is produced in and extruded from mesosomes of *Staphylococcus aureus*. It has been reported that lytic enzymes are excreted into the medium from *S. faecalis* var. zymogenes (23) which are thought to originate in the mesosome. These studies

provide some basis for speculation that the mesosomes of group D streptococci may be the site of production of the material which accumulates to form cores. Group D streptococci are pathogenic in humans, causing urinary tract infections, endocarditis, and wound infections (118) as well as a severe pyelonephritis in rats (46). Lacking any experimental evidence, it is difficult to speculate whether cores may have a role in the pathogenicity of group D streptococci.

Many structures in bacteria, such as spores, are produced only after active, logarithmic growth has ceased. Felter et al. (30) reported the same phenomenon in *Vibrio marinus* in relation to the production of intracellular, myelin-like membrane configurations.

One further, rather unlikely, speculation is that cores could represent spore-like structures that contain DNA and protect the cells from losing viability from the effects of the acid produced in the cultures.

The relationship between structure and function in microorganisms is little understood at this point, as evidenced by the work discussed previously in the literature review. An understanding of the functional capacities of cellular structures can be gained only through the combining of physiological, biochemical, and structural studies.

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BIOGRAPHICAL SKETCH

Sylvia Ethel Coleman was born March 23, 1933, in Gainesville, Florida. She graduated from Mainland High School in Daytona Beach, Florida, in June, 1951. In February, 1955, she received a Bachelor of Science degree and in August, 1956, a Master of Science degree with a major in bacteriology from the University of Florida. She lived in Paris, France, from 1956 to 1959 and studied at L'Alliance Française. She worked from 1959 to 1960 as a medical bacteriologist at Mound Park Hospital in St. Petersburg, Florida, and from 1960 to 1967 as a research microbiologist in the Research Laboratory of the Veterans Administration Center, Bay Pines, Florida. In September, 1967, she returned to the University of Florida and held a graduate teaching assistantship from September, 1967, to March, 1969, an NDEA Title IV fellowship from April, 1969, until August, 1970, and a graduate teaching assistantship from September, 1970, to December, 1972. In January, 1972, she began work as an electron microscope technician in the Ultrastructure Laboratory of the Division of Biological Sciences at the University of Florida.

In May, 1972, she was recipient of the award given by The Society of the Sigma Xi for outstanding accomplishment in research by a graduate student at the University

of Florida. She is a member of the American Society for Microbiology, the Southeastern Branch of the American Society for Microbiology, the Florida Academy of Sciences, the New York Academy of Sciences, and is an associate member of The Society of the Sigma Xi. She is currently a candidate for the Ph.D. degree to be awarded in August, 1972.

She is married to Al Herbert Bernstone of St. Petersburg, Florida, and Los Angeles, California.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Max. E. Tyler
Professor of Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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for Dean, College of Agriculture

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